Innate Immune Defence to *Helicobacter pylori*

By

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Abstract

Helicobacter pylori exhibits tropism for the human stomach causing a spectrum of complications ranging from gastritis to gastric cancer in susceptible individuals. The mechanism(s) that allow the bacteria to persist and cause disease are unfolding. β-defensins are a family of endogenous, epithelial anti-microbial peptides that engage in host defense most prominently at mucosal surfaces. We and others have previously shown that human β-defensin (hBD)-2 and -3 are potent bactericidal agents against *H. pylori*. At present the identity of signalling pathways involved in host-bacterial cross talk leading to modulation of host antimicrobial immunity are unknown.

The present study firstly investigated the potential role of bacterial virulence factors in mediating human β-defensin gene expression during *H. pylori* infection. AGS gastric epithelial cells were infected with cytotoxic *H. pylori* strains (60190, 84-183) and isogenic mutant strains (*cagA-*, *cagE-*, *vacA- and Cag*PAI-). Human β-defensin (hBD2 & -3) gene expression quantified by RT-PCR and β-defensin transcriptional regulation was followed by transient transfection studies utilising hBD2 and -3 promoter luciferase constructs. We found hBD2 induction was dependent upon an intact *cag*PAI and minimal involvement was observed for the bacterial virulence factors CagA and VacA in modulating β-defensin expression.

We sought to investigate the bacterial component responsible for instigating epithelial innate immune responses. Through the use of siRNA for NOD1 we determined a role for NOD1-dependent NF-κB activation in mediating hBD2 but not hBD3 expression. Experiments utilising specific inhibitors of the MAP Kinase pathways directed us to delineate the role of each pathway in modulating β-defensin expression by the activation of stably transfected conditional MAP Kinase mutants. These studies revealed critical involvement of ERK pathway in the regulation of hBD3 but not hBD2 gene expression.

Signalling upstream of ERK was explored and revealed EGFR as the host receptor responsible for detection and initiation of hBD3 gene and peptide production.

Our studies demonstrated a crucial role for NOD1 in *H. pylori*-mediated hBD2 but not hBD3 expression and implicate EGFR transactivation in mediating hBD3 but not hBD2 expression, thus indicating two distinct regulatory mechanisms at play during innate immune host response to *H. pylori* infection.

Publications and Abstracts

Publications

1. Boughan, P.K., Argent, R.H., Body-Malapel, M., Park, J.H., Ewings, K.E Bowie, A.G., Ong, S.J., Ewings, K.E., Cook, S.J., Sorensen, O.E., Manzo, B.A., Inohara, N., Klein, N.J., Nunez, G., Atherton, J.C., Bajaj-Elliott, M.
   Nucleotide-binding oligomerization domain-1 (NOD-1) and Epidermal Growth Factor Receptor (EGFR): Critical regulators of β-defensins during *H. pylori* infection. *Journal of Biological Chemistry,* 2006 Apr 28, 281 (17):11637-48.
2. Zilbauer, M., Dorrell, N., Boughan, P.K., Harris, A., Wren, B.W., Klein, N.J., Bajaj-Elliott, M.
   Intestinal Innate Immunity to *Campylobacter jejuni* Results in Induction of Bactericidal Human Beta-Defensins 2 and 3. *Infection & Immunity,* 2005 Nov; 73(11): 7281 - 7289.
3. George, J.T., Boughan, P.K., Karageorgiou, H., Bajaj-Elliott, M.
   Host anti-microbial response to *Helicobacter pylori* infection. *Molecular Immunology*, 2003 Nov; 40 (7):451-6.

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Oral presentation

1. Boughan, P. K., Argent, R.H., Bowie, A.G., Ong, S.J., Ewings, K.E., Cook, S.J., Sorensen, O.E., Manzo, B.A., Klein, N.J., Atherton, J.C., Bajaj-Elliott, M.
   NOD1 and EGFR: Central regulators of epithelial β-defensins in response to *Helicobacter pylori*
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1. Boughan, P. K., Argent, R.H., Bowie, A.G., Ong, S.J., Ewings, K.E., Cook, S.J., Sorensen, O.E., Klein, N.J., Atherton, J.C., Bajaj-Elliott, M.
   Modulation of gastric epithelial human β-defensins (HBD) 2 and -3 during *H. pylori* infection. *Helicobacter*, 2005 Oct;10 (5): 494
   European *Helicobacter* study group (EHSG) XVII International workshop gastrointestinal pathology and *Helicobacter*, Falconer Centre, Copenhagen, Denmark October 12-14 2005
2. E.I. Kazakos, A. Harris, S.J. Ong., P.K. Boughan., M. Curtis, M. Bajaj-Elliott.
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European *Helicobacter* study group (EHSG) XVII International workshop gastrointestinal pathology and *Helicobacter*, Falconer Centre, Copenhagen, Denmark October 12-14 2005

3. Zilbauer, M., Dorrell, N., Boughan, P.K., Harris, A., Wren, B.W., Klein, N.J., Bajaj-Elliott, M.
   ***Campylobacter jejuni* capsular polysaccharide and intestinal innate immunity.** *Journal of Paediatric Gastroenterology & Nutrition.* 2005 May; 40(5):651.
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   Contribution of MAPK signaling pathways to defensins gene regulation is stimuli-specific.
   Gordon Research Conference on Antimicrobial peptides, Ventura Beach Marriott, Ventura Beach, California, USA March 6-11 2005
5. Ong, S.J., Dixon, D., Boughan, P.K., Klein, N., Bajaj-Elliott, M.
   Defensins as Bactericidal agents against *Neisseria*
   Gordon Research Conference on Antimicrobial peptides, Ventura Beach Marriott, Ventura Beach, California, USA March 6-11 2005
6. Boughan, P. K., George, J.T., Argent, R.H., Atherton, J.C., Bajaj-Elliott, M.
   Regulation of Host Innate Immune Responses during *H. pylori* Infection *Clinical and Investigative Medicine,* 2004 Aug 27(4):168.
   12th International Congress of Immunology & 4th Annual Conference Federation of Clinical Immunological Societies, Montreal, Canada, July 18-23 2004
7. Zilbauer, M., Dorrell, N., George, J.T., Boughan, P.K., Bajaj-Elliott, M.
   The Role of Human beta-Defensins (hBDs) during *Campylobacter jejuni* Infection. *Clinical and Investigative Medicine,* 2004 Aug 27(4):168.
   12th International congress of Immunology & 4th Annual conference Federation of clinical Immunological societies, Montreal, Canada, July 18-23 2004
8. Zilbauer, M., Dorrell, N., George, J.T., Boughan, P.K., Bajaj-Elliott, M.
   The Role of Human Beta Defensins during *Campylobacter jejuni* Infection. *Journal of Pediatric Gastroenterology & Nutrition,* 2004 June; 39 (1):S80.
9. Boughan, P. K., George, J.T., Thomas, R., Atherton, J.C., Bajaj-Elliott, M.
   Regulation of Host Innate immune responses during *H. pylori* Infection. *Gastroenterology,* 2004 Apr;126 (2):A1-815.
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    Regulation of Defensin Gene Expression during *H. pylori* Infection.
    12th International Workshop on *Campylobacter, Helicobacter* and related organisms, Aarhus, Denmark, 6-10 September 2003.
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Abbreviations

AMPs	**Antimicrobial peptides**
AP-1	**Activated protein-1**
bp	**Base pair**
CagA	**Cytotoxin associated gene A**
Cag A	**Cytotoxin associated protein A**
***Cag*PAI**	**Cytotoxin associated gene pathogenicity island**
cDNA	**Complementary DNA**
dNTPs	**deoxynucleotide triphosphates**
DTT	**Dithiothreitol**
DU	**Duodenal ulcer**
EGFR	**Epidermal Growth Factor Receptor**
FCS	**Foetal calf serum**
GAPDH	**Glyceraldehyde-3 phosphate dehydrogenase**
GI	**Gastrointestinal**
H. pylori	***Helicobacter pylori***
hBD	**Human beta defensin**
HD	**Human defensin**
HEK	**Human Embryonic Kidney**
HNP	**Human neutrophil defensin**
HP-NAP	***Helicobacter pylori* neutrophil-activating protein**
HRP	**Horse radish peroxidase**
4-HT	**4-Hydroxytamoxifen**
IFNγ	**Interferon gamma**
IL	**Interleukin**

IL-1RA	**Interleukin-1 Receptor Antagonist**
IRAK	**Interleukin-1 Receptor -associated kinase**
KO	**Knock-out**
L	**Litre**
LeX	**Lewis X antigen**
LPS	**Lipopolysaccharide**
MALT	**Mucosa-associated lymphoid tissue**
µL	**micro-Litre**
µM	**micro-Molar**
MAPK	**Mitogen Activated Protein Kinase**
mBD4	**Murine Beta Defensin 4**
min	**Minute**
mL	**Milli-litre**
MMLV-RT	**Moloney murine leukaemia virus- Reverse Transcriptase**
mRNA	**Messenger ribonucleic acid**
MyD88	**Myeloid differentiation factor 88**
NapA	**Neutrophil activating gene**
NF-κB	**Nuclear factor-κB**
nM	**nano-Molar**
NOD	**Nuclear-binding Oligomerisation Domain**
OD	**Optical density**
PAGE	**Poly-acrylamide Gel Electrophoresis**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PRR	**Pattern Recognition Receptor**
RT	**Reverse Transcription**

secs	**Seconds**
SDS	**Sodium-Dodecyl-Sulphate**
TBS	**Tris buffered saline**
TIR	**Toll/Interleukin-1 Receptor**
TLR	**Toll-Like Receptor**
TNFα	**Tumour necrosis factor α**
TRAF-6	**TNF Receptor-associated factor 6**
vacA	**Vacuolating cytotoxin gene A**
VacA	**Vacuolating cytotoxin protein A**

CHAPTER 1

Introduction

1.0 *Helicobacter pylori*

1.1. Discovery of a *Campylobacter*-like organism

Marshall and Warren were the first to report the presence of *Campylobacter*-like organism in the stomach lining of patients with chronic gastritis and peptic ulcers (Marshall and Warren, 1984). In 1984, the name *Campylobacter pyloridis* was proposed and urease production by the bacterium was established. The presence of urease allowed the detection of the bacterium in gastric biopsies (McNulty and Wise, 1986) and even today forms the basis of clinical diagnostic tests.

Comparison of *C. pyloridis* gene sequences with other members of the *Campylobacter* family led to the bacterium being designated to a new genus called *Helicobacter* and *C. pyloridis* was referred to as *Helicobacter pylori* (Maddocks, 1990). Completion of the genome sequences of two *H. pylori* strains 26695 and J99 (Alm *et al.*, 1999) has allowed more detailed microbiological characterisation of the organism.

H. pylori is a spiral shaped, Gram-negative, microaerophilic bacillus, but coccoidal forms have also been reported under a variety of conditions including nutrient deprivation and antibiotic stress (Figure 1.1). Both forms are believed to be infectious, the clinical significance of the coccoid form remains unclear.

1.2 Pathogenesis and Clinical Associations

Helicobacter pylori is a highly successful extracellular mucosal pathogen that colonises the gastric epithelium causing persistent inflammation (chronic active gastritis). It exhibits selective tropism for the gastric mucosa or sites of gastric metaplasia and does not colonise regions of the stomach undergoing intestinal metaplasia (Figure 1.2; Ernst *et al.*, 2006, Peterson, 1991, Suerbaum and Michetti, 2002). It infects up to 50% of the population worldwide, yet only a proportion of susceptible individuals develop clinical complications

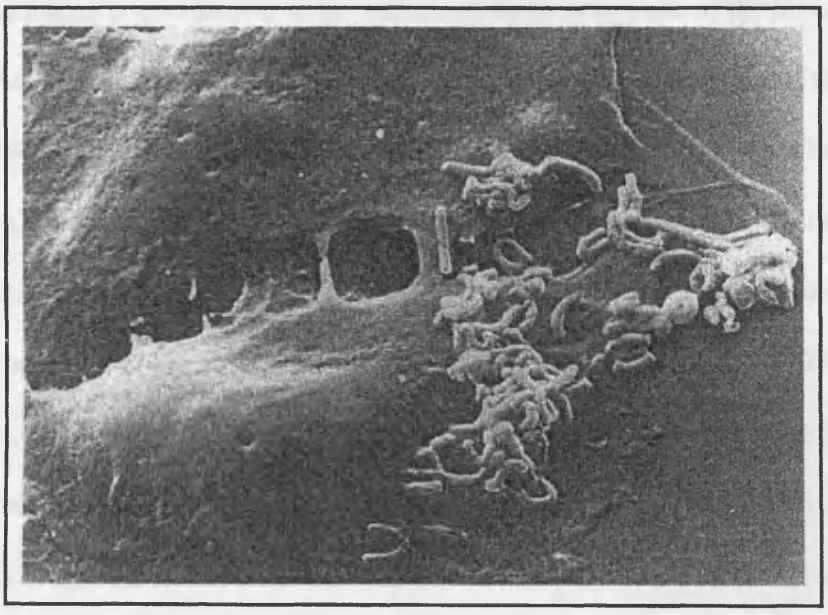


Figure 1.1: *H. pylori* (spiral and coccoidal) adhering to gastric epithelium (from Marais *et al.*, 1999).

Figure 1.2: Histological identification of *H. pylori* in a gastric mucosal biopsy. Arrows indicate the presence of *H. pylori* adjacent to the gastric epithelium (from Ernst *et al.*, 2006).

(Montecucco and Rappuoli, 2001). Variation observed in clinical outcome of *H. pylori* infection is now known to be influenced by the virulence of the infecting strain (Figure 1.3; Atherton *et al.*, 1997a, Blaser and Crabtree, 1996) susceptibility of the host (El-Omar *et al.*, 2000) and environmental factors (Blaser and Atherton, 2004). The clinical course of *H. pylori* infection ranges from peptic ulceration to distal gastric adenocarcinoma and gastric mucosal lympho-proliferative diseases such as MALT lymphoma (Nomura *et al.*, 1991, Parsonnet *et al.*, 1994, Peterson, 1991, Suerbaum and Michetti, 2002).

A correlation between the pattern and distribution of gastritis and subsequent complications has been noted. Patients with antral-predominant gastritis (the most common form) are predisposed to duodenal ulcers, whereas patients with corpus-predominant gastritis and multifocal atrophy are more predisposed to gastric ulcers, gastric atrophy, intestinal metaplasia and ultimately gastric carcinoma (Suerbaum and Michetti, 2002). The majority of gastric and duodenal ulcers result from *H. pylori* infection and the lifetime risk of development of peptic ulcer for *H. pylori* carriers varied from 3% in United States to 25% in Japan (Schlemper *et al.*, 1996).

Gastric infection with *Helicobacter pylori* causes mucosal inflammation that is concentrated in the gastric antrum. This pattern of inflammation is referred to as type B gastritis and although type B gastritis is most intense in the antrum, it commonly coexists with a less severe inflammation in the fundus. Almost all (95%) patients who have this pattern of gastritis will concurrently be infected with *H. pylori* (Blaser and Atherton, 2004). The mechanism regarding formation of duodenal and gastric metaplasia is believed to be in response to acid exposure, thus leading to direct epithelial damage or secondary inflammation. This acid release derives from parietal cells resulting from histamine binding to histamine-type 2 receptors. The histamine is released after G-cells produce gastrin (Dockray *et al.*, 2001).

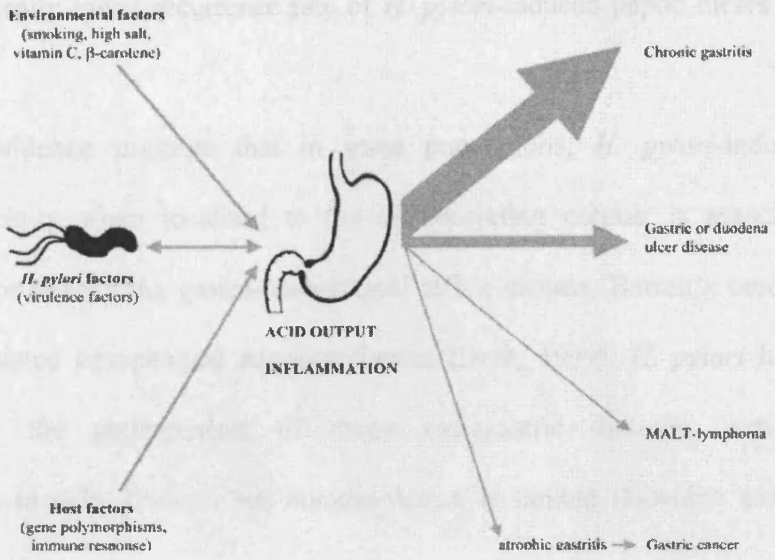


Figure 1.3: Contribution of factors influencing *H. pylori* disease outcome (taken from van Amsterdam *et al*, 2006).

These injured duodenal areas may heal by acquiring gastric epithelium, which might be better adapted than duodenal epithelium for acid exposure. After gastric metaplasia has occurred, these areas are likely to provide a site for bacterial adherence, thus contributing to the pathogenesis of duodenal ulceration.

Carcinoma of the stomach is the second most common cause of cancer death worldwide (Montecucco and Rappuoli, 2001) and implication of *H. pylori* as a key risk factor for the development of gastric carcinoma has been recognized through many clinical studies, first described by Parsonnet *et al* in 1991. As a result, *H. pylori* has been classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) in 1994. Most notably, mucosa-associated lymphoid tissue (MALT) lymphomas account for approximately 5% of all gastric cancers (Lacy and Rosemore, 2001), and is particularly significant as it usually goes into complete remission upon eradication of the pathogen (Bayerdorffer *et al*., 1995). This is also true for peptic ulcers as eradication of the organism

caused a drastically lower recurrence rate of *H. pylori*-induced peptic ulcers (Marshall *et al.*, 1988).

Conversely, evidence suggests that in some populations, *H. pylori*-induced chronic gastritis, especially when localised to the acid-secreting corpus, is associated with a reduced risk for developing gastro-oesophageal reflux disease, Barrett's oesophagus, and Barrett's-associated oesophageal adenocarcinoma (Dent, 1999). *H. pylori* has also been implicated in the pathogenesis of many extragastric diseases, extending from atherosclerosis to skin diseases but documentation is limited (Howden and Leontiadis, 2000).

1.3 Epidemiology and Transmission

Incidence of *Helicobacter pylori* infection is worldwide; however prevalence varies amongst age groups, ethnic groups and among countries. For example, approximately 30% of the developed countries and up to 80% of the developing world is infected (Figure 1.4; Atherton, 1997a). Overall occurrence is strongly correlated with socioeconomic conditions (Malaty and Graham, 1994), poor sanitation and hygiene being major risk factors. Most infections appear early in life, with infection rate independent of gender (Lacy and Rosemore, 2001). In adults of higher socioeconomic groups the prevalence of seropositivity increases with age to eventually reach 40% or more; and this increase is due to low but cumulative risk of acquisition in Western countries in past years than a recent event, representing cohort effect (Cullen *et al.*, 1993).

In industrialised countries direct transmission from person to person *via* vomitus, saliva, or faeces is the predominant route of transmission, whereas additional routes such as water may be important in developing countries (Goodman *et al.*, 1996, Parsonnet *et al.*, 1999). Despite the stomach being the natural habitat, *H. pylori* has been found in dental plaque

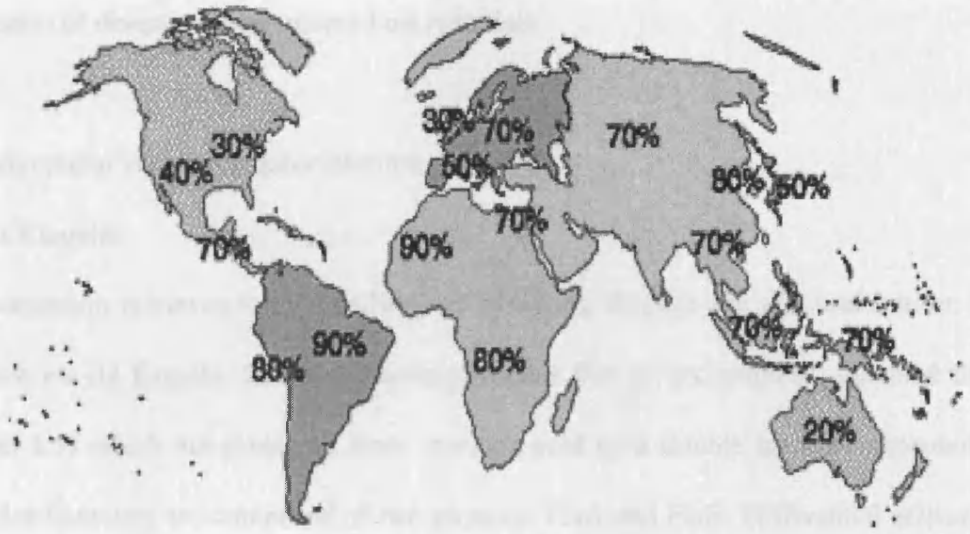


Figure 1.4: Epidemiology of *H. pylori* infection.

The prevalence of *H. pylori* colonisation correlates with socio-economic status (*Helicobacter* foundation website, 2004)

and saliva (Nguyen *et al.*, 1995). Although there is no evidence to suggest zoonotic transmission, the bacterium has been detected in nonhuman primates and other animals (Dore *et al.*, 2001, Handt *et al.*, 1994).

1.4 Contribution of bacterial and host factors to disease pathogenesis

H. pylori is very much adapted to the ecological niche of the stomach. Despite the acid environment, peristalsis and the induction of local immune responses the organism colonises and can persist in this micro-environment for decades (Valle *et al.*, 1996). Bacterial virulence factors aid the pathogen not only to penetrate the mucous layer but also to allow attachment to the epithelial cells and furthermore, evade the host immune response

to permit colonisation (Blaser and Atherton, 2004). The bacterium is also highly competent in DNA uptake from other strains (Suerbaum *et al.*, 1998) and therefore, there is continual generation of diversity to overcome host restraints.

1.4.1 Bacterial virulence determinants

1.4.1.1 Flagella

The bacterium achieves rapid motility and spreading through the mucosal barrier of the stomach *via* its flagella. Each bacterium possesses five or six uniploar, sheathed flagella (Figure 1.5) which are protected from stomach acid by a double layer of phospholipids. Flagellar filaments are composed of two proteins, FlaA and FlaB. Differential activation of the two genes suggests composition of the flagella may vary depending on dictating environmental conditions (Josenhans *et al.*, 1995).

Figure 1.5: *H. pylori* - a gastric pathogen.

(Courtesy of Dr Andrew Harris, Bart's and the Royal London Medical School, UK)

1.4.1.2 Urease

Urease is essential for the virulence and successful colonisation of the bacterium (Eaton *et al.*, 1991). Animal studies have shown that urease-negative *H. pylori* mutants do not colonise the gastric mucosa (Tsuda *et al.*, 1994). The enzyme breaks down urea (present in gastric juice and extracellular fluid) into ammonia and bicarbonate (Israel and Peek, 2001). Ammonia alkalinises the microenvironment to allow *H. pylori* to survive in gastric acid and colonise (Mobley, 2001). Enzyme activity is regulated by a unique pH-gated urea channel, UreI, which opens at low pH and increases permeability of the bacterial membrane to urea but prevents influx of urea under neutral condition (Weeks *et al.*, 2000).

1.4.1.3 Adhesins

The majority of *H. pylori* colonising gastric mucosa are free-living, with an approximate 20% binding intimately to gastric epithelia (Clyne and Drumm, 1993). BabA is a well-characterised bacterial ligand involved in host cell recognition and binding. BabA is a 78kDa membrane-bound adhesin encoded by the gene *babA2* and it binds to fucosylated Lewis B blood group antigen present on epithelium (Ilver *et al.*, 1998). *H. pylori babA2*$^{+}$ strains are linked to an increased risk for duodenal ulceration and gastric adenocarcinoma, whereas strains deficient in babA2 are more likely to cause gastritis alone (Gerhard *et al.*, 1999). These findings have been verified in a transgenic mouse model expressing Lewis B antigen (Guruge *et al.*, 1998). Recent studies have demonstrated that selection of BabA variants with increased or decreased adherence to Lewis B antigens may play a role in host adaptation (Aspholm-Hurtig *et al.*, 2004, Solnick *et al.*, 2004).

Although BabA is the most studied adhesin, several other adhesion-related factors are likely to play a role in bacterial adherence. Absence of HopZ leads to decreased bacterial binding (Peck *et al.*, 1999). HpaA (HP0410) binds to host sialylated glyconjugates (Evans *et al.*, 1993), AlpA and AlpB are also significant adhesins (de Jonge *et al.*, 2004, Odenbreit

et al., 2002), which unlike BabA are found in nearly all bacterial strains, implying a contributory role in bacterial colonisation (Odenbreit *et al.*, 1999).

1.4.1.4 Lipopolysaccharide (LPS)

Lipopolysaccharides comprise a major component of the cell wall of Gram-negative bacteria. The low immunological and endotoxic activity of *H. pylori* LPS has been confirmed both in animal and cell culture models (Ferrero, 2004, Smith *et al.*, 2003). Despite reduced activity, *H. pylori* LPS does demonstrate some effects on gastric mucosa. *H. pylori* LPS from type I strains can induce apoptosis in guinea-pig gastric cells (Kawahara *et al.*, 2001) and induce endoethelin and TNF-α (Ierardi *et al.*, 2003). *H. pylori* LPS affects mucin synthesis and pepsinogen secretion, which may further contribute to the pathogenesis of *H. pylori* related diseases (Moran *et al.*, 1996).

LPS represents an important component of Gram-negative bacteria. It comprises a polysaccharide O-antigen chain (a core oligosaccharide) and a lipid moiety (lipid A) that anchors the LPS molecule to the outer membrane (Raetz and Whitfield, 2002). Unlike other Gram-negative bacteria, *H. pylori* post-translationally modifies its O-antigen by undergoing fucosylation, hence displaying mimicry to Lewis antigens present on human epithelial cells (Monteiro *et al.*, 2000). Detailed mechanistic studies have revealed *H. pylori* LPS variation in fucosylation patterns allow the micro-organism to evade innate and adaptive host immunity (Nilsson *et al.*, 2006). An example of this subversion is highlighted by examination of a pattern recognition receptor, surfactant protein D (SP-D) which binds directly to LPS of Gram-negative bacteria via the carbohydrate recognition domain (Kuan *et al.*, 1992). SP-D is present in the gastric mucosa and expression increases in individuals infected with *H. pylori* (Murray *et al.*, 2002). Further analysis of the interaction of SP-D with *H. pylori* LPS revealed the ability of O-antigen modifications to evade innate immune recognition, contributing to bacterial persistence (Moran *et al.*, 2005).

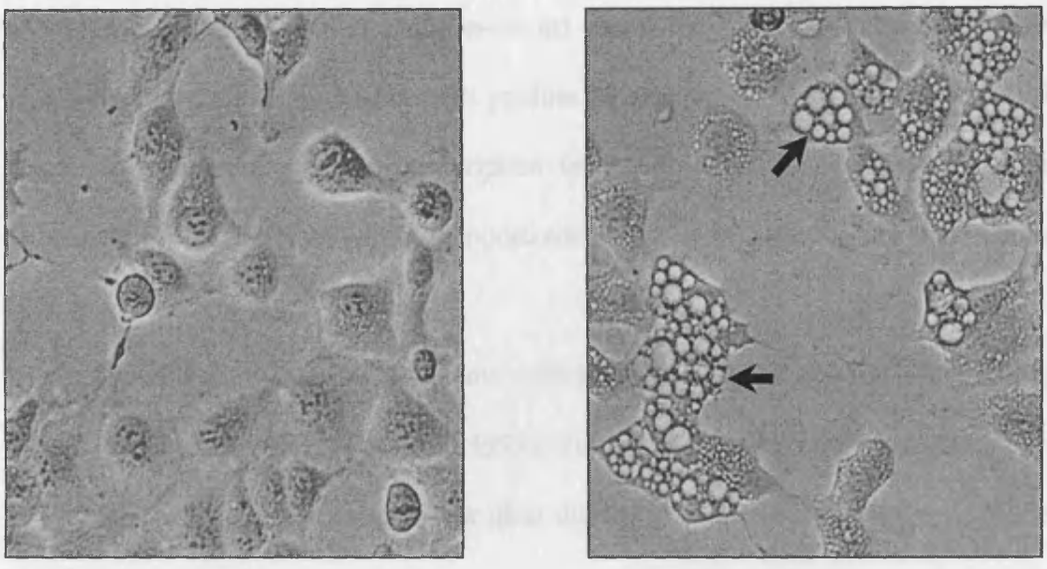
1.4.1.5 *Helicobacter pylori*- Neutrophil Activating Protein (HP-NAP)

One of the hallmarks of *H. pylori* infection is extensive neutrophil infiltration and ensuing tissue damage (Fiocca, 1994, Satin, 2000). *Helicobacter pylori*- Neutrophil Activating Protein (HP-NAP) is a 150kDa dodecameric iron-binding protein that adheres to human neutrophils and endothelial cells leading to production of reactive oxygen radicals (Evans *et al.*, 1995, Yoshida, 1993). Recombinant HP-NAP exhibits chemotactic activity for human neutrophils and monocytes, a potential mechanism *H. pylori* may utilise to modulate host phagocytic activation (Satin, 2000). All *H. pylori* strains investigated to date contain the gene encoding HP-NAP, although protein levels show marked variation (Evans and Harmon, 1995). Clinical and pathogenic consequences of HP-NAP protein levels remain unclear.

1.4.1.6 Vacuolating cytotoxin (VacA)

VacA is a secreted 95kDa exotoxin which is expressed in an active form by approximately 50% of *H. pylori* strains (Leunk *et al.*, 1988). This multimeric pore-forming protein induces vacuole formation in cultured epithelial cells (Figure 1.6) and causes epithelial damage when administered to mice (Telford *et al.*, 1994). Vacuoles arise in the perinuclear area and enlarge in size to fill the entire cytosol, eventually leading to death by necrosis (Figura *et al.*, 1989). This vacuolar degeneration of host cells is achieved by interference of intracellular membrane fusion and the vacuoles produced appear to be a hybrid between lysosomal and late endosomal compartments (Covacci *et al.*, 1999).

Low pH allows the disassociation of the inactive oligomeric VacA protein into active monomers, the latter being resistant to both acid and pepsin. These biochemical properties emphasise the toxin's suitability to the gastric milieu (de Bernard *et al.*, 1995). The



(a) (b)

Figure 1.6: Effects of vacuolating toxin on AGS gastric epithelial cells (a) untreated cells; (b) VacA treated cells.

(Courtesy of Prof J.C Atherton, University of Nottingham, UK)

exotoxin inserts itself into the epithelial membrane forming hexameric anion-selective, voltage dependent channels, allowing release of cytosolic bicarbonate and organic anions (Szabo *et al.*, 1999). Furthermore, VacA toxin modifies tight junctions, increases permeability for iron, nickel and other nutrients, essential for *H. pylori* growth and urease activity (Montecucco and Rappuoli, 2001). In addition to vacuole generation, VacA also affects cells directly *in vitro* by inducing cytoskeletal changes, apoptosis, and suppression of epithelial cell proliferation and migration (Cover *et al.*, 2003, Kuck, 2001, Pai *et al.*, 1999). All strains possess the *vacA* gene but there is significant strain-dependent sequence variation in the VacA protein particularly in the mid-region (m1 or m2) and in the signal sequence (s1a, s1b or s2). *H. pylori* strains carrying different combinations of both regions have been isolated (Atherton *et al.*, 1995, Letley and Atherton, 2000). Strains expressing

s1/m1 generally secrete high protein levels, m1 strains are more active than m2, s1a type is more effective than s1b and s2 doesn't produce detectable activity (Atherton *et al.*, 1997b, Blaser and Atherton, 2004). Transcription for *vacA* induction occurs in proximity to epithelial cells *in vitro*, highlighting importance for VacA in bacterial-host cell interactions (van Amsterdam *et al.*, 2003).

Clinical studies show 30-40% of patients suffering from ulceration do not possess toxigenic strains (Figura *et al.*, 1989, Tee *et al.*, 1995). Further studies have concluded that a *vacA* s1 genotype confers greater likelihood for ulcer disease than an s1b or s2 genotype (Atherton, 1997b).

1.4.1.7 *Cytotoxin- associated gene* Pathogenicity Island

Of all bacterial virulence factors implicated in disease pathogenesis, the *Cytotoxin-associated gene* pathogenicity island (*Cag*PAI) is the most prominent (Censini *et al.*, 1996). *H. pylori* strains are termed *cag*+ (virulent) or *cag*– (less virulent) depending on the presence of this 40kb genomic fragment region, comprising approximately 30 genes (Censini *et al.*, 1996). The role of *cag*PAI in disease pathogenesis has been confirmed in the Mongolian gerbil model of infection, where *cag*+ *H. pylori* caused severe inflammation, and further gastric complications typical of *H. pylori* infection, compared to animals colonized with *cag*- strains which were affected with mild asymptomatic gastritis (Ogura *et al*, 2000).

The pathogenicity island encodes a type IV secretion apparatus, homologous to those found in other bacteria, including the Ti plasmid transfer system of *Agrobacterium tumefaciens* and the pertussis toxin secretion machinery of *Bordetella pertussis* (Covacci and Rappuoli, 2000). This export machinery is required for translocating the bacterial cytotoxin-associated antigen, CagA, protein into host cells (Odenbreit *et al.*, 2000). Although *cagA* is often used as a marker for the whole *cag*PAI, recent evidence suggests the presence of

cagA does not necessarily correlate with an intact *cag*PAI (Censini *et al.*, 1996, Covacci *et al.*, 1993, Salama *et al.*, 2000). While this immunodominant protein is present in 60-70% of *H. pylori* strains (Akopyants *et al.*, 1998), it is prevalent in 50-60% of patients suffering from gastritis and in 88-100% of bacterial isolates in patients with duodenal ulceration (Covacci *et al.*, 1993).

Cellular investigations into the function of CagA, a 128-145kDa protein showed the injected protein on entering host cells was rapidly phosphorylated (Asahi *et al.*, 2000, Segal *et al.*, 1999). Phosphorylated CagA allows recruitment of SHP-2 (a tyrosine phosphatase) to the cellular membrane, leading to actin polymerization and pedestal formation (Covacci and Rappuoli, 2000). The net result of these molecular interactions is a distinct change in the phenotype (humming-bird) of the infected gastric epithelia (Segal *et al.*, 1999). Phosphorylated CagA has been implicated in increased frequency of gastric cancer cases where patients are infected with *cag*+ strains (Covacci and Rappuoli, 2000). Specifically, *H. pylori* strains inducing higher levels of CagA phosphorylation, owing to increased number of tyrosine phosphorylation motifs (TPMs), are more likely to be associated with gastric cancer (Argent *et al.*, 2004). Proto-oncogenes *c-fos* and *c*-jun are known to be activated in gastric epithelial cells infected with *cag*+ strains and may correspond to a critical step in the development of neoplasia (Meyer-ter-Vehn *et al.*, 2000).

1.4.2 Genetic Host factors

As discussed above, there are several bacterial determining factors influencing the outcome of *H. pylori*-related diseases. Studies investigating the effect of strain variation have revealed that even the most virulent (CagA+, *Cag*PAI+) *H. pylori* strains can be harboured by asymptomatic individuals (Ernst *et al.*, 2006). This indicates the important contribution of host factors in modulating pathogenesis and disease outcome (El Omar *et al.*, 2000, El Omar *et al.*, 2001, El Omar *et al.*, 2003, Kim *et al.*, 2006).

Polymorphisms in host cytokine genes such as IL-1β, IL-1 receptor antagonist (IL-1RA), IL-10 and TNFα have been linked to gastric cancer progression (Chakravorty *et al.*, 2006, El Omar *et al.*, 2000, El Omar *et al.*, 2003, Machado *et al.*, 2001, Wu *et al.*, 2003, Yea *et al.*, 2001). Specifically, a polymorphism (a C-T base transition) at position -31 in the *IL-1B* gene results in gastric cancer in approximately 31% of the study population. Functionally, this polymorphism induced a 5-fold induction in transcriptional activity of factors regulating IL-1β production in response to LPS *in vitro* (El Omar *et al.*, 2000).

Polymorphisms may lead to an increase in pro-inflammatory (IL-1β, TNFα) cytokines or inadequate anti-inflammatory (IL-10) cytokine release which can result in gastric mucosal damage owing to a Th1 biased inflammatory response (Eaton *et al.*, 2001, El Omar *et al.*, 2003). This response affects acid secretion as the cytokines IL-1β and TNFα prevent parietal cells from producing acid (Beales and Calam, 1998, Padol and Hunt, 2004, Takashima *et al.*, 2001), which in turn may allow persistence and spread of *H. pylori* (Bjorkholm *et al.*, 2004).

Presence of multiple cytokine polymorphisms and infection demonstrate a synergistic increase in risk of gastric cancer (El Omar *et al.*, 2003). Additionally, in a study of African subjects, IFNγR1 polymorphisms were implicated in determining susceptibility of individuals to *H. pylori* infection (Thye *et al.*, 2003), which may aid toward reasoning the high prevalence of infection but low incidence of cancer (the 'African enigma') (Holcombe, 1992).

1.5 Host Innate Immunity

Although it is clear that *H. pylori* possesses and utilises several virulence determinants to ensure its survival, the bacterium is also able to manipulate host immune responses to allow persistence in the stomach. For example, by the induction of certain antimicrobial peptides

such as β-defensin 2 and LL-37, *H. pylori* may attempt to defend its gastric niche from other potential pathogens or limit its own proliferation so it may control nutrient acquisition. Other mechanism(s) by which *H. pylori* subverts host immunity are discussed below.

1.5.1 Epithelial cells

The establishment of NF-κB activation by *H. pylori* infection has since led to studies detailing specific signalling components involved in this signalling cascade, most notably the identification of Nucleotide-binding Oligomerisation Domain-1 (NOD1) as the host pattern recognition receptor (Hirata *et al.*, 2006, Hirata *et al.*, 2006, Maeda *et al.*, 2000, Mori *et al.*, 2003). Although, both IκB kinase (IKK) and NF-κB-inducing kinase (NIK) are key upstream signal transduction players implicated in *H. pylori*-mediated NF-κB activation (Hirata *et al.*, 2006, Maeda *et al.*, 2000, Mori *et al.*, 2003, Viala *et al.*, 2004), involvement of the adaptor protein myeloid differentiation factor 88 (MyD88) and tumour necrosis factor α-associated factor 6 (TRAF6) remains controversial, as one study suggests no role for TRAF6 in NF-κB-mediated Regulated upon Activation, Normal T cells Expressed and Secreted (RANTES) expression (Mori *et al.*, 2003) but other studies implicate TRAF6 in *H. pylori*-mediated NF-κB activation (Hirata *et al.*, 2006, Maeda *et al.*, 2000). *H. pylori*- mediated signalling pathways involved in NF-κB activation are a subject of active research.

1.5.2 Other innate cells

Epithelial cells provide the first line of defence and form a critical component of innate defence. It is well established that IL-8 produced by epithelial cells in response to *H. pylori* chemoattracts neutrophils to the site of infection. Activated neutrophils present in the inflamed gastric mucosa produce reactive oxygen and nitrogen species which cause damage to host tissue (Crabtree, 1996). *H. pylori* avoids being harmed by free radicals by producing

enzymes involved in reactive oxygen intermediates (McGee and Mobley, 1999) and by doing so the bacterium is able to prevent microbicidal effects in addition to causing tissue damage.

Macrophages produce iNOS (inducible nitric oxide synthase), in response to *H. pylori* leading to cell injury and apoptosis (Wilson *et al.*, 1996). Nitric Oxide (NO) is known to exhibit microbicidal effects. As bacterial expressed arginase and iNOS share the substrate, L-arginine, *H. pylori* manages to escape killing by NO by regulating NO synthesis (Gobert *et al.*, 2001). Macrophages exposed to an isogenic mutant lacking arginase were found to be more susceptible to killing by NO compared to wild type strain (Baldari *et al.*, 2005). This avoidance of bactericidal activity of macrophages clearly provides at least one mechanism by which *H. pylori* is able to subvert host immunity.

1.6 Infection models

In order to strengthen *in vitro* findings and obtain a clearer view of host immune responses *in vivo*, several animal infection models have been investigated. Recent transcriptional profiling analyses in murine models have revealed gastric cells respond to *H. pylori* infection by induction of inflammatory genes including IL-1β and RANTES (Mueller *et al.*, 2004). Furthermore, similar transcriptional profiling studies in the rhesus macaque model resulted in significant induction of immune and inflammatory genes such as β-defensin 2, chemokine receptor 2 and protease inhibitor 3 (Huff *et al.*, 2004). These studies provide valuable insight into the programmed transcriptional response to bacterial infection *in vivo.*

Transgenic mice and specified genotypes make murine models an obvious choice for studying *H. pylori* pathology *in vivo,* unfortunately wild type mice are not as susceptible to *H. pylori,* and infection does not lead to pathology as observed in human disease (Court *et al.*, 2002). A murine pathogen, *H. felis* has replaced *H. pylori* in many studies as it causes

more severe inflammation (Court *et al.*, 2002, Sakagami *et al.*, 1996). This strain, however, lacks many important virulence determinants such as the *cag*PAI and VacA (Ernst *et al.*, 2006) highlighting limitations of such studies.

In contrast to mouse models, the Mongolian gerbil is more susceptible to *H. pylori* infection, causing gastric pathology similar to that observed in human disease (Crabtree *et al.*, 2004, Ogura, 2000, Peek, Jr. *et al.*, 2000, Watanabe *et al.*, 1998, Zheng *et al.*, 2004). Progression from colonisation to gastric adenocarcinoma in gerbils has been limited to studies from Japan and China (Peek, Jr. and Crabtree, 2006) and a recent study investigating the potential of certain strains becoming more carcinogenic after passages *in vivo* yielded interesting findings (Franco *et al.*, 2005). A single colony from a gerbil infected with *H. pylori* strain B128 for 3 weeks was used to infect independent gerbils. Despite the parent gerbil not presenting with gastric adenocarcinoma, 75% of the newly colonised gerbils had gastric adenocarcinoma 8 weeks post-infection (Franco *et al.*, 2005). These results demonstrate the ability of *H. pylori* to cause gastric cancer in the Mongolian gerbil model and therefore highlight a suitable model to study disease pathogenesis.

1.7 Adaptive Immunity

Although the gastric epithelium is the main point of microbial contact for the extracellular *H. pylori*, increasing impairment of epithelial integrity during on-going infection, must allow the bacterium to interact with underlying antigen-presenting cells and phagocytes. Dendritic cells (DCs) are potent antigen-presenting cells and important mediators between the innate and acquired immune system, to date there have been few studies investigating DC maturation in response to *H. pylori* infection (Guiney *et al.*, 2003, Kranzer *et al.*, 2004, Voland *et al.*, 2003). Stimulation of DCs with *H. pylori* results in IL-6, IL-8, IL-10 and IL-12 production (Guiney *et al.*, 2003, Kranzer *et al.*, 2004) and further investigations suggest

*Cag*PAI and VacA independent mechanism(s) of activation and maturation are operative (Kranzer *et al.*, 2005).

1.7.1 Cell-mediated immunity

There are two functional subsets of CD4+ T lymphocytes, T helper 1 (Th1) and T helper 2 (Th2), which have distinct cytokine profiles. Th1 cells are known to enhance cell-mediated immunity whereas production of Th2 cells induce B cell activation and differentiation (humoral immunity) (Israel and Peek, Jr., 2006). Although a novel, pathogenic Th-17 T cell subset has been identified recently (Veldhoen and Stockinger, 2006), no studies investigating the role of this subset in *H. pylori* induced disease have yet been reported.

Th1 type responses are stimulated in response to most intracellular bacteria whereas Th2 responses are induced by extracellular pathogens (Portal-Celhay and Perez-Perez, 2006). Considering this, one would expect Th2 type responses to *H. pylori* to predominate since it is largely a non-invasive bacterium, but this is not the case. *H. pylori* infection in both humans and animal models exhibits a predominant Th1 profile, with increased levels of IFNγ (Bamford *et al.*, 1998, Crabtree *et al.*, 2004). The cytokine expression profiles have been documented in gastric mucosa of infected *H. pylori* individuals including children (Luzza *et al.*, 2001). Interestingly, a recent study exploring *H. pylori*-mediated effects on IFNγ signalling revealed disruption of signal transducer and activator of transcription 1 (STAT1) signalling (Mitchell *et al.*, 2004). Another strategy by which *H. pylori* achieves Th1 polarisation may be by suppression of IL-4, a Th2 cytokine, by a *cag*PAI and Vac independent mechanism (Ceponis *et al.*, 2003). By interfering with host signalling, the bacterium seems to manipulate a Th1 rather than Th2 bias and successfully evade complete clearance.

The above mechanism(s) have not identified bacterial factors responsible for the Th1 profile; however, a very recent investigation implicates HP-NAP in directly modifying host

immunity (Amedei *et al.*, 2006). HP-NAP increased IFNγ, reduced IL-4 in addition, neutrophil and monocyte dependent induction of IL-12 and IL-23 was noted, resulting in an overall Th1 cytokine profile (Amedei *et al.*, 2006). This Th1 bias was also observed *in vivo* in human gastric mucosa in response to *H. pylori* infection.

1.7.2 Antibody immunity

H. pylori infection results in vigorous IgG and IgA antibody production both in serum and gastric mucosa (Bhat *et al.*, 2005, Crabtree *et al.*, 1993, Luzza *et al.*, 1995) but despite this, infection still persists. *In vitro* phagocytosis and killing of *H. pylori* by polymorphonuclear leukocytes was enhanced by binding to IgG (Tosi and Czinn, 1990). *H. pylori* activates and is susceptible to both the classical pathway, even without antibodies, and the alternative pathway of complement (Berstad *et al.*, 2001, Gonzalez-Valencia *et al.*, 1996). An investigation into the role of mannan-binding lectin (MBL), which also promotes phagocytosis and activates complement by the lectin pathway found no association between MBL deficiency and *H. pylori* gastritis (Bak-Romaniszyn *et al.*, 2006).

Infection studies in B cell deficient mouse models show that initial bacterial colonisation is similar in both wild type and B cell K/O mice. In contrast, prolonged infection results in clearance of bacteria with more severe gastritis in B cell K/O mice but not in wild type litter (Akhiani *et al.*, 2004), suggesting the presence of antibodies does not confer protection but rather are detrimental to successful bacterial elimination.

Further studies from the same researchers utilising IL-10 and IgA deficient mice revealed markedly less colonisation when compared to wild type mice, which re-iterates earlier findings that antibodies do not eliminate infection, allowing colonisation and persistence (Akhiani *et al.*, 2005). These investigations provide conclusive evidence that antibody production does not confer protection against *H. pylori* but rather the presence of IgA in particular, enables the pathogen to evade host immune responses.

1.8 Vaccines

Despite *H. pylori* being a human pathogen, some strains have been adapted to allow studies in animal models. Mice and gerbils have been particularly useful in vaccine studies (Del Giudice *et al.*, 2001). Vaccines to date have been based on urease, CagA, VacA, NAP and whole cell killed bacteria (Corthesy-Theulaz *et al.*, 1995, Hatzifoti *et al.*, 2004, Hatzifoti *et al.*, 2006, Keller and Michetti, 2001).

The oral route of vaccine administration in humans has proved to be most effective in the stimulation of antigen-specific IgA antibody responses in the stomach and duodenum when compared in trials to intranasal, intrajejunal, or rectal (Johansson *et al.*, 2004). Oral immunisation of urease with an adjuvant, heat-labile enterotoxin of *Escherichia coli* (LT), resulted in marked decrease in colonisation of *H. pylori* in the gastric mucosa of infected individuals (Michetti *et al.*, 1999). This finding demonstrates that although eradication of the bacterium was not achieved, this vaccine was immunogenic in this cohort of patients. Another vaccine study comprising formalin-inactivated whole cell bacteria also with the LT adjuvant exhibited effects on both infected and non-infected individuals (Kotloff *et al.*, 2001), as proliferation and production of IFNγ from Peripheral Blood Mononuclear Cells (PBMCs) was observed. All vaccination studies to date show reduced colonisation but not eradication of the organism.

1.9 Host epithelial innate defence to *H. pylori*

The pathogenesis of *H. pylori*-mediated infection and inflammation is most likely to be initially triggered by the direct interaction of the bacterium with the gastric epithelial cell layer (Figure 1.7). Although recent studies have shown that *H. pylori* has the ability to enter epithelial cells (Kwok *et al.*, 2002, Semino-Mora *et al.*, 2003) the bacterium remains

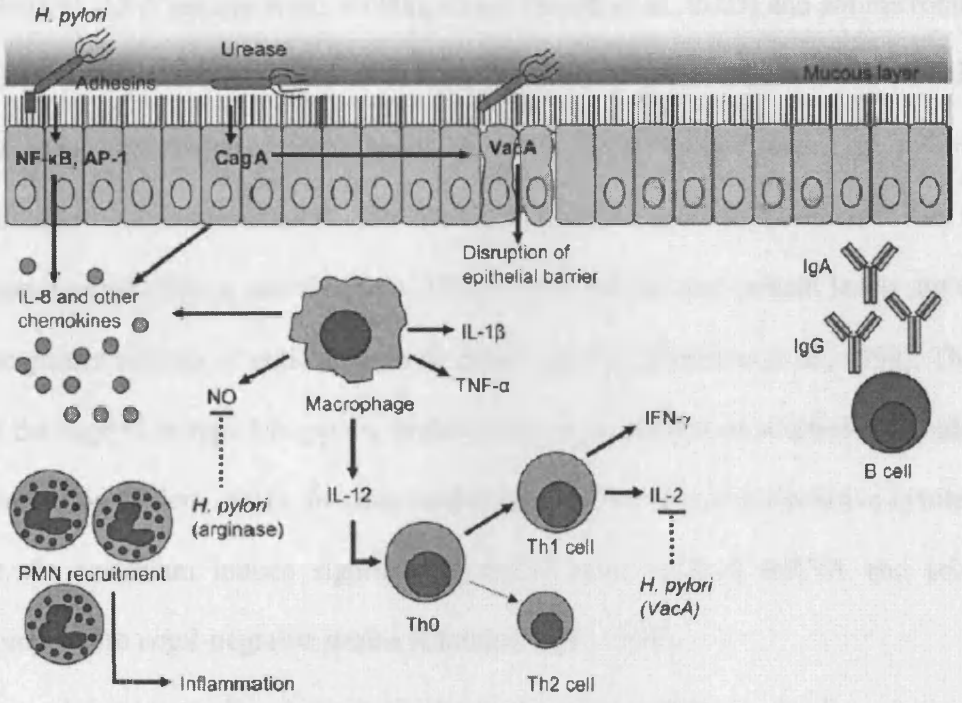


Figure 1.7 Schematic representation of the stomach mucosa colonised by *Helicobacter pylori*, showing the main virulence factors involved in colonisation and disease (from Portal-Celhay and Perez-Perez, 2006).

largely non-invasive adhering to the cell surface (Corthesy-Theulaz *et al.*, 1996, Hazell *et al.*, 1986). At present our understanding of why this non-invasive bacterium elicits a potent pro-inflammatory response leading to severe tissue damage remains unclear. Further, the mechanism(s) that allow the bacterium to persist in the presence of a robust immune response are unknown (Bodger and Crabtree, 1998). It is becoming increasingly clear that bacterial infection can stimulate the gastro-intestinal (GI) epithelium to actively participate

in host defence (Dommett *et al.*, 2005). During the inflammatory process, the epithelium expresses increased levels of innate defence genes including IL-8, ICAM-1 (Crowe *et al.*, 1995), IL-18 (Crabtree *et al.*, 1999a), Cox-2 (Smith *et al.*, 2003) and antimicrobial peptides (Bajaj-Elliott *et al.*, 2002, O'Neil *et al.*, 1999, Wada *et al.*, 1999, Hase *et al.*, 2003).

Amongst host innate defence genes known to be modulated during *H. pylori*-mediated infection and inflammation, the role and regulation of IL-8 remains the most well characterised (Blaser and Crabtree, 1996). IL-8 mRNA and protein levels are elevated in the gastric mucosa of patients with *H. pylori* gastritis (Crabtree *et al.*, 1994). The presence of the *cag*PAI in type I *H. pylori* strains is known to present an additional stimulus for IL-8 production (Allen, 2001). *In-vitro* studies have shown that *cagA*-positive cytotoxic strains of the bacterium induce significantly higher level of IL-8 mRNA and protein when compared to *cagA*-negative strains (Crabtree *et al.*, 1994).

Several *in-vivo* studies suggest similar higher IL-8 expression in the presence of type I strain compared to type II strains (Peek, Jr. *et al.*, 1995). The increased IL-8 expression observed in *cagA*-positive *H. pylori* strains seems to be related to bacterial density (Atherton *et al.*, 1997a). Yamoaka and co-workers explored the relationship between *cagA* positive *H. pylori* duodenal ulcer (DU) and non-DU patients (Yamaoka *et al.*, 1999). The ability of *cagA* positive *H. pylori* to induce IL-8 *in vitro* was similar and independent of whether the strain was from a patient with DU or gastritis. In the antrum, IL-1β and IL-8 production was closely related to bacterial density. DU patients exhibited higher bacterial density and cytokine levels but there was no evidence of enhanced virulence of *H. pylori* between the two groups of patients.

Bauditz and colleagues were the first to measure cytokine levels in both *H. pylori* positive and negative gastritis (Bauditz *et al.*, 1999). On the basis of the degree of inflammation the authors found no significant difference between the levels of pro-inflammatory cytokines

such as IL-1β, TNF-α and IL-8. These findings suggest that the presence of pro-inflammatory cytokines and chemokines is a non-specific host response to inflammation which can occur in the absence of pathogen. However in the same study the levels of IL-12 were significantly increased in the *H. pylori*-positive group.

1.10 Antimicrobial peptides

Antimicrobial peptides (AMPs) are an evolutionarily ancient arsenal providing protection in all multicellular organisms (Zasloff, 2002a, Zasloff, 2002b). Host AMPs mediate the first line of defence against pathogenic insult. Since the discovery of the first antimicrobial peptide by Boman and colleagues (Steiner *et al.*, 1981) more than 500 peptides have been identified.

In humans, two main classes of AMP families, defensins and cathelicidins are found in a wide variety of tissues with predominant expression in the epithelium and in circulating phagocytes (Table 1.1; Zasloff, 2002a, Zasloff, 2002b). Other mammalian AMPs including histatins (Devine, 2003), dermicidin (Schittek *et al.*, 2001) and anionic peptide (Brogden *et al.*, 1997) are restricted to a few animal species and tissues. A single animal can contain different classes of peptides and a number of variants in a given class. The diversity of AMPs reflects the magnitude of host responses required to interact with the microbial world. AMPs exhibit a broad range of activity against a range of microbes including Gram-negative and Gram-positive bacteria, fungi, protozoa and viruses (for recent reviews see Eckmann, 2005, Finlay and Hancock, 2004, Ganz, 2005, Lehrer, 2004, Selsted and Ouellette, 2005, Klotman and Chang, 2006).

1.11 Defensins

Mammalian defensins are endogenous antibiotics that play a critical role in host innate defence. The two main classes of human defensin genes, α and β, differ in their disulphide

Defensin				
Conventional name	**Type**	**Cell source**	**Synthesis**	**Release**
HNP1 ~4	α	Neutrophils	Constitutive	Degranulation
		CD8 T cells	Inducible	Secretion?
HD5 ~6	α	Paneth cells	Constitutive	Degranulation
Mouse cryptidin	α	Paneth cells	Constitutive	Degranulation
hBD1	β	Keratinocytes and epithelial cells	Constitutive and inducible	Secretion
hBD2 ~4	β	Keratinocytes and epithelial cells	Inducible	Secretion
BNDBs	β	Neutrophils	Constitutive	Degranulation
Bovine TAP	β	Epithelial cells	Inducible	Secretion
RTD-1	θ	Neutrophils, monocytes	Constitutive	Degranulation
Cathelicidins		Neutrophils, keratinocytes, epithelial cells, mast cells, and monocytes/macrophages	Constitutive and inducible	Degranulation or secretion
*Abbreviations: HNP, human neutrophil peptide; HD, human defensin; hBD, human β-defensin; BNDB, bovine neutrophil-derived β-defensin; TAP, tracheal antimicrobial peptide; RTD-1, rhesus theta defensin-1.				

Table 1.1 Cell sources and regulation of mammalian defensins and cathelicidins (from Yang *et al*, 2004).

pairing, genomic organisation and in their tissue distribution (Table 1.1, Figure 1.8; Froy, 2005, Ganz, 2003, Jia *et al.*, 2001, Selsted and Ouellette, 2005). A third class of defensin, θ defensin, has been isolated from macaque monkey leukocytes (Tang, 1999). θ defensins have evolved in primates but are inactive in humans due to mutations encoding premature stop codon (Cole, 2002). Primate θ defensins possess lectin-like properties which may contribute to their ability to protect cells from HIV-1 infection (Wang *et al.*, 2003a).

1.11.1 Gene structure and tissue distribution

Human α-defensins are expressed predominantly in neutrophils where they comprise 5-7% of total protein content of the azurophilic granules (Ganz and Lehrer, 1998). Neutrophil α-defensin peptides (HNP1-4) are known to be responsible for the non-oxidative killing properties of these phagocytes. For many years, HNPs were thought to be expressed solely in polymorphonuclear leukocytes, however, recent studies suggest that monocytes and natural killer cells also express HNP1-3 (Chalifour *et al.*, 2004, Mackewicz *et al.*, 2003). Paneth cells of the small intestine express two members (HD-5 and -6) of the α-defensin family (Bevins, 2006, Jones and Bevins, 1992, Mallow *et al.*, 1996). Increasing evidence suggests a potential role for intestinal peptides in maintaining low bacterial count in the small intestine, in protecting pluripotent stem cells and in fighting potential pathogens (Bevins *et al.*, 1999, Cunliffe, 2003, Dommett *et al.*, 2005). In addition, the presence of HD-5 in the human female reproductive tract (Selsted and Ouellette, 2005) indicates a contribution of this peptide in maintenance of a sterile environment in the reproductive tract. Human β-defensins are a second, closely related family of AMPs. These AMPs are distributed in epithelia of a large variety of tissues (Table 1.1). To date six members (hBDs) of this family have been characterised, although bioinformatics suggests the presence of potential 30 hBDs and 45 murine β-defensin genes (Morrison *et al.*, 2003, Schutte *et al.*, 2002, Semple *et al.*, 2003, Semple *et al.*, 2005).

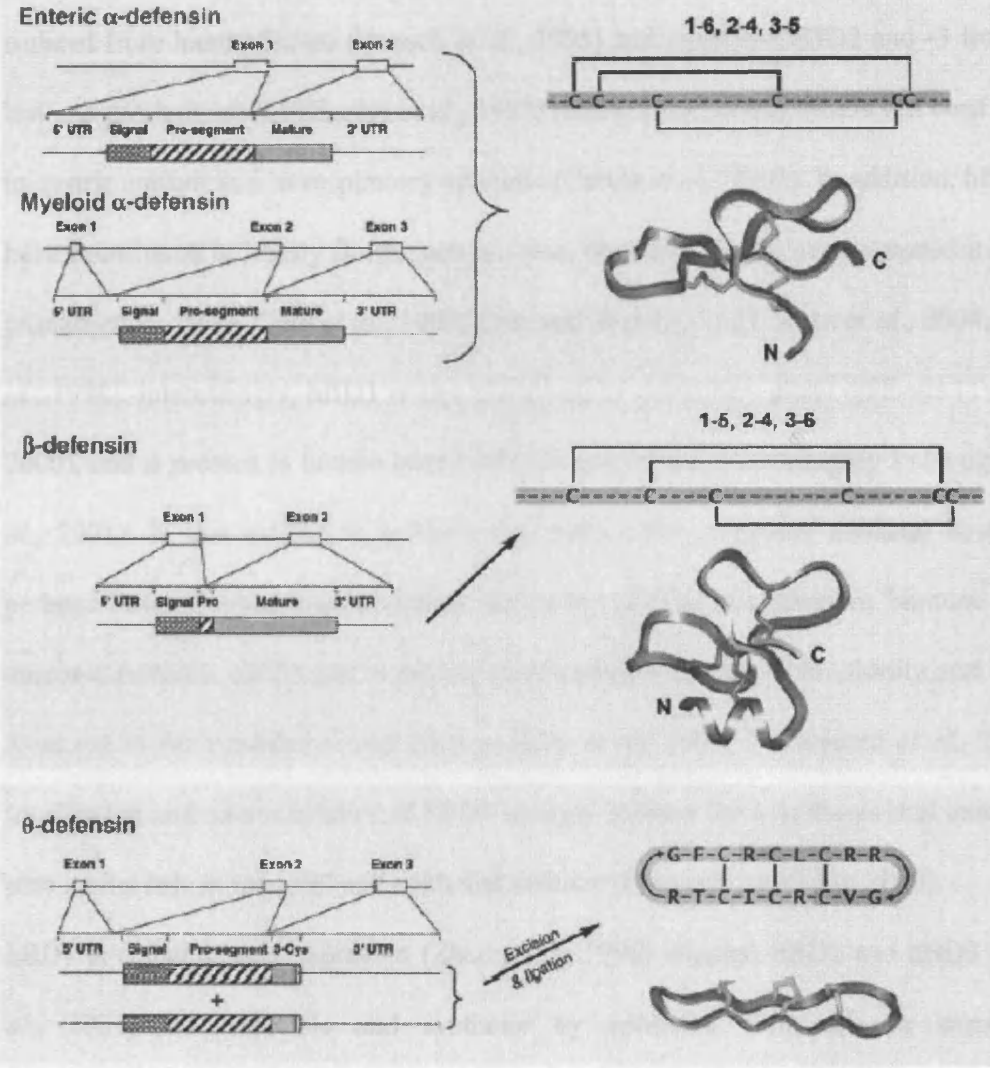


Figure 1.8 Representative gene and peptide structures of mammalian defensins (from Selsted and Ouellette, 2005). The left panel aligns α-, β-, θ-defensin genes with corresponding signal, pro-segment and mature defensin sections. The right panel highlights differing structural arrangements, in particular the position of the three disulphide bonds.

Tracheal antimicrobial peptide (TAP) was the first β-defensin detected in bovine airway epithelia (Diamond *et al.*, 1991). The first human β defensin (hBD)-1 was originally isolated from haemofiltrate (Bensch *et al.*, 1995) and inducible hBD2 and -3 from human lesional psoriatic scales (Harder *et al.*, 1997, Harder *et al.*, 2001). hBD4 has been identified in gastric antrum and in respiratory epithelia (Garcia *et al.*, 2001). In addition, hBD1 and 2 have been found in bodily fluids such as urine, bronchial fluids, nasal secretions and saliva (Abiko *et al.*, 2003, Cole *et al.*, 1999, Cole and Waring, 2002, Ross *et al.*, 2004, Valore *et al.*, 1998). Interestingly, hBD1 is also expressed by mammary gland epithelia (Tunzi *et al.*, 2000), and is present in human breast milk in concentrations of roughly 1–10 μg/ml (Jia *et al.*, 2001). In this setting, it is likely that hBD-1 may augment neonatal host defences perhaps through antimicrobial effects and/or by priming the adaptive immune system at mucosal surfaces. hBD5 and 6 are the most recent members of the family and have been localised to the epididymis and airways (Kao *et al.*, 2003, Yamaguchi *et al.*, 2002). The localisation and characteristics of hBDs strongly support the hypothesis that these peptides play a vital role in mucosal and epithelial defence (Hancock and Scott, 2000).

hBD1 is constitutively expressed (Zhao *et al.*, 1996) whereas hBD2 and hBD3 (Harder *et al.*, 2001) are inducible and synthesis by epithelial cells can be stimulated by microorganisms and by proinflammatory cytokines e.g. TNFα and IL-1. hBD2 has been widely investigated and is augmented by infection in the gastrointestinal tract, keratinocytes, oral epithelia, respiratory tract and most other mucosal surfaces both *in vitro* and *in vivo* (Bajaj-Elliott *et al.*, 2002, George *et al.*, 2003, Harder *et al.*, 1997, Hiratsuka *et al.*, 1998, Lehmann *et al.*, 2002, Liu *et al.*, 1998, O'Neil *et al.*, 1999, Singh *et al.*, 1998), hBD3 and -4 expression studies to date are limited.

Both defensin families reside on chromosome 8, studies by Dorin and colleagues and McCray and co-workers have assigned the murine and human β-gene locus to 8p22-23

region, although several newly identified β-defensins are located on chromosomes 6 and 20 (Schutte *et al.*, 2002, Semple *et al.*, 2005). Sequence analyses suggest that both α and β-defensin families arose by gene duplication and divergent evolution (Jia *et al.*, 2001, Maxwell *et al.*, 2003, Semple *et al.*, 2003). Defensins are encoded by two exons which code for a precursor pre-propeptide. This includes an N-terminal signal sequence, an anionic pro-segment and a C-terminal cationic segment that on cleavage yields the active peptide (Lehrer and Ganz, 2002). Exon 1 encodes the signal sequence and the pro and mature peptides are coded by the second exon. Although both neutrophil and Paneth cell α-defensins undergo proteolytic cleavage to yield active peptide (Eckmann, 2005), the processing between the two cell types is markedly different (Bajaj-Elliott, 2003). In neutrophils, the peptides are stored in their active form (Ganz, 1985). HD-5 and -6 are also synthesised as large molecules but in contrast to HNPs are stored in precursor form (Ghosh *et al.*, 2002). Studies by Bevins and colleagues suggest close proximity of pro HD-5 precursor molecule to trypsinogen in Paneth cell granules under resting conditions. Upon bacterial stimulation, trypsinogen is converted to trypsin, the enzyme responsible for converting HD-5 to its active form (Ghosh *et al.*, 2002). Why such diverse mechanisms for α-defensins processing exist in nature is unknown. Sequence analyses of β-defensins shows the signal peptide is followed by a very short pro-piece with active molecule residing at the C-terminus. The short size of the pro-piece suggests that this sub-family of defensins are likely to be secreted directly without an intermediate storage step. However, no studies to date have investigated the kinetics of β-defensin peptide expression and secretion, to confirm the above hypothesis.

1.11.2 Protein Structure

Defensins share the general structural hallmark of AMPs i.e. they are small, (28-45 amino acids) peptides with molecular weight ranging between 3-5kDa (Fellermann and Stange,

2001, Niyonsaba and Ogawa, 2005). α and β-defensins differ in their disulphide linkage patterns; α-defensins have three intramolecular bonds positioned at C1-C6, C2-C4 and C3-C5, whereas β-defensins form C1-C5, C2-C4 and C3-C6 (Figure 1.8; Ganz and Lehrer, 1998). Despite the variation in S-S linkage both peptides adapt a similar β-sheet secondary structure framework. The tri-disulphide folded structure is required for microbicidal activity of HNPs as linearised peptides are ineffective against viruses and bacteria (Daher *et al.*, 1986, Mandal and Nagaraj, 2002). Interestingly, more recent data suggests that the primary function of the disulphide bridges is to prevent proteolysis in protease-rich effector sites, such as enzyme-rich environment of the GI tract (Selsted and Ouellette, 2005), as mutations in all three disulphide bridges in hBD3 resulted in bactericidal activity similar or greater than the native peptide (Wu *et al.*, 2003). Similarly, native cryptdin-4, a mouse Paneth cell α-defensin, but not its disulphide bond analogs is resistant to protease degradation whilst none lose their antibacterial activity (Maemoto *et al.*, 2004).

1.11.3 Mechanism of Action

Most AMPs are cationic molecules with spatially spaced hydrophobic regions. The peptides are small (<100 amino-acids) with an excess of positive charge due to lysine and arginine residues and are approximately 50% hydrophobic (Hancock and Scott, 2000). A general mechanism by which the peptides are able to kill bacteria is by depolarising and permeabilising membranes (Figure 1.9), but there are some examples in which the lethal target is cytoplasmic (Devine and Hancock, 2002). In Gram-negative bacteria, the peptides can associate with the negatively charged phospholipids of the outer membrane or directly bind and replace the divalent magnesium ion binding site involved in interacting with LPS. These modifications lead to expansion of the outer membrane causing disruption of function and integrity. Such structural changes allow the passage of large antibiotics and peptides themselves into the interfacial region of the inner membrane, where on reaching

sufficient concentrations the peptides aggregate within the membrane leading to pore formation. Gram-positive bacteria do not have an outer membrane, but AMP binding to outer wall components such as lipoteichoic acid (LTA) suggests a different mode of action. All defensins characterised to date have direct antimicrobial activity (Ganz, 2003, Ganz and Lehrer, 1998, George *et al.*, 2003, Lehrer, 2004, Lehrer and Ganz, 2002, Schroder, 1999) in the μM range however, there are differences in potency between the recombinant β-defensin peptides (George *et al.*, 2003, Zilbauer *et al.*, 2005). β-defensins and LL-37 (the only Cathelicidin family member expressed in man) show synergy against *S. aureus* and *E. coli* and this synergy was further enhanced in acidic conditions (Chen *et al.*, 2005). The α-defensins HNP1-4, are only active at concentrations higher than 10^{-5} M and exhibit broad range of activity, killing a range of microbes (Ganz, 2003, Yang *et al.*, 2004).

The first molecular interaction between cationic AMP and the anionic microbial cell membrane is primarily electrostatic. High concentrations of salt are competitively inhibitory to this interaction, however, activity of hBD3 and θ-defensins is not usually dampened (Harder *et al.*, 2001). The first mechanism is the formation of ion pores within the membrane (Figure 1.9), and the second model, "carpet model" has been suggested in the case of hBD2 specifically (Hoover *et al.*, 2000). Electrostatic forces pull apart the peptide molecules into the membrane and the molecules accumulate in a 'carpet'. The membrane is strained and the peptides shift in arrangement to relieve this strain, thereby forming pores or "wormholes" in the membrane (Huang, 2000, Lohner *et al.*, 1997). Despite microbial membrane depolarisation and permeabilisation being the most accepted mechanism of defensin action, other mechanisms cannot be ruled out, such as induction of microbial autolytic enzymes (Sahl *et al.*, 2005).

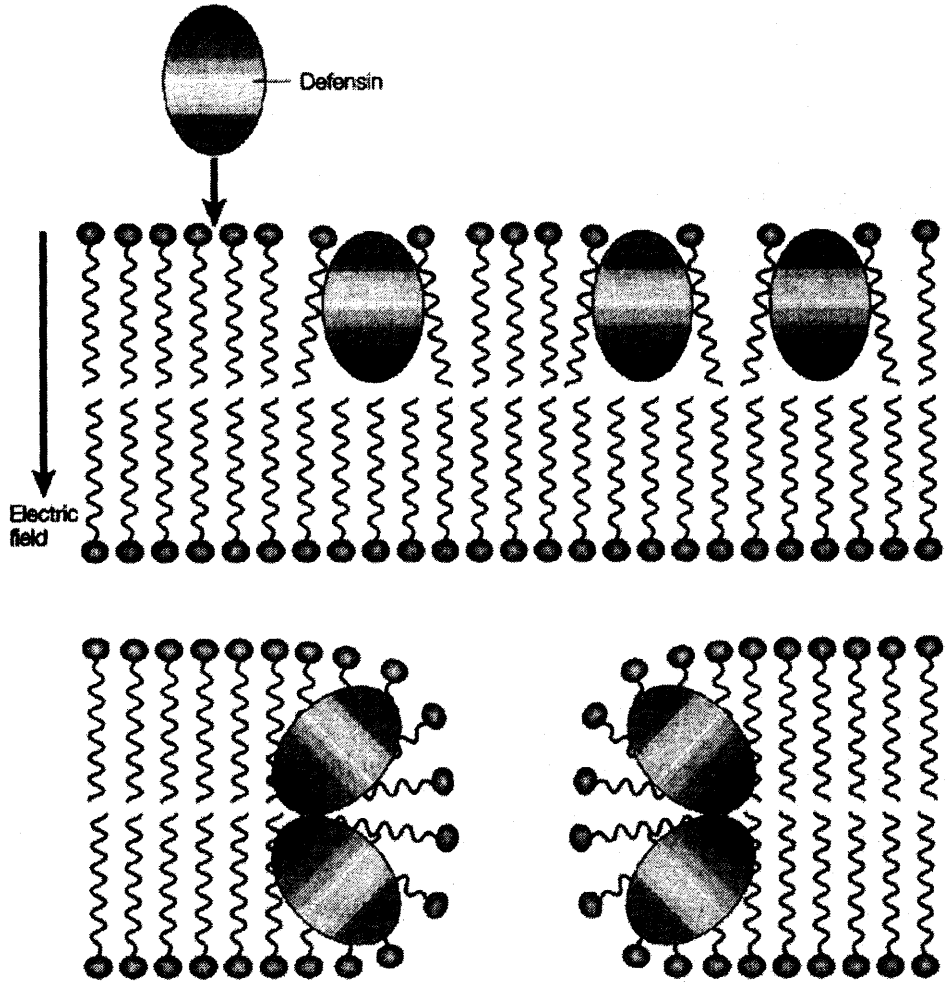


Figure 1.9 The carpet–wormhole model of action of defensins. Most defensins (shown as large ovals) are amphipathic molecules that have clusters of positively charged amino-acid side chains and hydrophobic amino-acid side chains. This allows them to interact with microbial membranes, shown schematically with their negatively charged phospholipid headgroups and hydrophobic fatty acid chains (from Ganz, 2003).

The antibacterial activity of hBD2 against *H. pylori* has been assessed by Hamanaka and colleagues as determined by co-incubation of varying concentrations of recombinant hBD2 with the bacteria followed by plating out the test samples. hBD2 was bactericidal in the micromolar range with complete eradication of the organism at higher peptide concentrations (Hamanaka *et al.*, 2001). hBD3 was recently shown to be up-regulated in response to *H. pylori* infection in gastric epithelial cells (AGS) and further evaluation of the antimicrobial potency of hBD1-3 conducted in a similar assay revealed that hBD3 was most potent of the hBDs, reducing survival down to 5% in the micromolar range, followed by hBD2 and finally hBD1 (George *et al.*, 2003).

LL-37 has also been implicated in *H. pylori* infection as significant induction of this peptide was observed in epithelial cells and gastric secretions of *H. pylori*-infected patients (Hase *et al.*, 2003). LL-37 exhibited specific anti-bacterial effects in the micromolar range. Induction of LL-37 was dependent upon an intact *Cag*PAI suggesting that the secretion system may play a part in eliciting host antimicrobial response (Hase *et al.*, 2003).

As multiple AMPs are likely to be present simultaneously at the mucosal surface during an infectious episode, synergy between the peptides may allow for more effective bactericidal activity against *H. pylori* and some synergy has been observed between the hBDs, as well as lysozyme or LL-37 (George *et al.*, 2003, Hase *et al.*, 2003). These results concur with those obtained in studies investigating synergistic activities of hBDs, LL-37 and lysozyme against *E. coli* and *S. aureus* (Chen *et al.*, 2005). Collectively, these findings suggest a significant role for the hBDs, in particular the highly potent antimicrobial hBD3, in *H. pylori*- mediated disease.

1.11.4 Physiological function

In vivo physiological antimicrobial effector function for defensins has been examined by the use of transgenic and knock-out mice. Mice lacking matrilysin, MMP-7, the enzyme

required for converting inactive α-defensin pro-form to active peptide, were impaired in their ability to clear *E. coli* and were more susceptible to *S. typhimurium* infection, when compared to control wild type litter (Wilson *et al.*, 1999). Salzman and colleagues observed increased protection of transgenic mice expressing HD-5 against *Salmonella* infection when compared to control litter, suggesting an important role for this peptide in mucosal defence of the GI tract (Salzman *et al.*, 2003).

β-defensin *in vivo* function has also been explored. Deletion of murine β-defensin-1 gene (*Defb1*) results in defective clearance of *Haemophilus influenzae* from the lung (Moser *et al.*, 2002). Another study showed increase in *Staphylococci* species in the bladders of *Defb1* K/O mice (Morrison *et al.*, 2002). An *in vivo* study found increased protection from *Bordetella pertussis* infection in the presence of exogenously administrated porcine β-defensin (pBD1) (Elahi *et al.*, 2006). There was also a suggestion of pBD1-mediated anti-bacterial activity against several porcine pathogens, demonstrating β-defensins as potential targets for anti-pertussis therapy.

Although the bactericidal function of defensins is well-established, recent studies implicate these peptides in novel functions including signalling to cells involved in adaptive immunity (Oppenheim *et al.*, 2003). Chemotactic activity of defensins occurs at nM concentration versus μM required for antimicrobial activity. The chemotactic properties include HNP1-3, hBD3 and -4 recruiting monocytes (Garcia *et al.*, 2001, Garcia *et al.*, 2001, Territo *et al.*, 1989), with hBD2 chemoattracting mast cells (Niyonsaba *et al.*, 2002). hBD1-3 are also chemotactic for immature dendritic cells (iDCs) (Yang *et al.*, 1999,Yang *et al.*, 2002). Murine BD2 mediates this interaction via binding to the chemokine receptor CCR6 present on DCs and T cells (Biragyn *et al.*, 2002). Recent advances in bioinformatical searches for novel β-defensin like peptides followed by *in situ* studies have revealed expression of some of these peptides is limited to epithelial epididymis. It has

been proposed that these novel members are not involved in innate defence but may serve physiological roles such as maturation of sperm cells of the male reproductive tract (Rodriguez-Jimenez *et al.*, 2003). This would not, however, necessarily exclude any indirect effects on host defence. Defensins have also been implicated in epithelial and fibroblast cell division (Murphy *et al.*, 1993), and enhancement of wound closure (Aarbiou *et al.*, 2004).

1.11.5 Role in Health and Disease

The relationship between defensins and infectious disease pathogenesis is a topic of active research. Antimicrobial activity of defensins is inhibited in respiratory epithelia in cystic fibrosis, owing to high mucosal salt concentration, which significantly contributes to increased susceptibility to chronic infection (Goldman *et al.*, 1997). The recurrent pulmonary infections were deduced to be due to hBD1 inactivation in the presence of salt (Smith *et al.*, 1996). Increased defensin levels have also been observed in other inflammatory lung diseases, such as diffuse panbroncheolitis (DPB), idiopathic pulmonary fibrosis (IPF) and acute respiratory distress syndrome (ARDS), indicating a potential role for defensins in the pathogenesis of these diseases, although any precise involvement still remains to be elucidated (Aarbiou *et al.*, 2002).

High expression of hBD2 has been found in inflamed skin epidermal cells, whereas keratinocytes from healthy skin do not express hBD2 mRNA expression (Liu *et al.*, 1998). This correlates well with high amounts of hBD2 peptide in lesional psoriatic scale extracts (Schroder and Harder, 1999) and a corresponding decreased incidence of several types of infection, only 7% of patients with psoriasis acquire skin infections compared to approximately 30% with atopic dermatitis (Christophers and Henseler, 1987). This decreased occurrence of skin infections in psoriasis has been confirmed to be due to elevated levels of both hBD2 and LL-37 (Ong *et al.*, 2002). So, a deficiency in expression

of hBD2 may account for the increased susceptibility of recurrent infections for patients suffering from atopic dermatitis. Further investigation suggests involvement of IL-10 dependent mechanism(s), as anti-IL-10 treatment of dermatitis skin explants enhanced the expression of hBD2 (Howell *et al.*, 2005). Also, IL-4 and IL-13 have been implicated in suppressing TNFα and IFNγ induced hBD3 induction (Nomura *et al.*, 2003), implicating inflammatory processes in atopic dermatitis as important features involved in gene regulation.

In the gastrointestinal tract, *Shigella* infection leads to modulation of antimicrobial peptides and host defence. In both children and adults experiencing *Shigella*-driven diarrhoea, synthesis of colonic hBD1 (in enterocytes) and LL-37 was noticeably suppressed (Islam *et al.*, 2001). This down-regulation may be a virulence determinant in disease progression. Up-regulation of hBD gene and peptide expression in response to infection with another pathogen causing diarrhoea, *C. jejuni* has also been observed (Zilbauer *et al.*, 2005). The mechanism(s) involved in this induction is an active area of research, as the identity of bacterial factors responsible for these effects remains elusive. Furthermore, several probiotic bacterial strains including *E. coli* strain Nissle 1917, cause potent induction of hBD-2 in intestinal epithelial cells, in an NF-κB-dependent manner (Wehkamp *et al*, 2004), confirming a role for β-defensins in intestinal innate defence.

Importantly, study into the expression of β-defensins in Inflammatory Bowel disease suggests increase of hBD2 and -3 in ulcerative colitis, with impaired expression in Crohn's disease (Wehkamp *et al*, 2003). These findings implicate β-defensins in providing an effective mucosal barrier function in humans, suggesting disruption in Crohn's disease is likely to lead to increased susceptibility to bacterial invasion, thus contributing to disease pathogenesis.

The co-localisation of an intracellular peptidoglycan receptor, NOD2 and α-defensins in the specialised Paneth cell has led to the notion that α-defensins may contribute to the pathogenesis of Crohn's ilietis, a chronic Inflammatory Bowel disease (Wehkamp *et al.*, 2005, Bevins, 2005, Bevins, 2006, Grimm and Pavli, 2004). Interestingly, it has also been postulated that NOD2 mediates β-defensin expression and the promoter activity was severely impaired in the presence of mutated NOD2 (Voss *et al.*, 2006), indicating a likely role for both defensin families in this condition.

The epithelium plays an active role in innate immune responses through the secretion of inflammatory cytokines, chemokines and antimicrobial peptides. One may hypothesise that host cell pattern recognition receptor signalling in response to microbes leads to protection due to induction and expression of β-defensins. These peptides may contribute to limiting pathogenic infection or indeed elimination of invading microorganism(s). In addition, defensins attract DCs and T cells leading to recruitment of adaptive immune cells.

An important issue facing the clinical management of *H. pylori* is the increasing prevalence of resistance to the antibiotic component of the present regimens. Identification of specific resistance mechanism(s) employed by *H. pylori* and our understanding of the regulation of naturally occurring antibiotics during infection and inflammation may provide insights into strategies to develop new therapeutic agents.

Aims & Hypothesis

The hypothesis to be investigated in this thesis is that *Helicobacter pylori* strains that establish chronic mucosal infection are armed with virulence factors that directly or indirectly (via host inflammatory mediators) modulate expression and function of epithelial β-defensins. Such strains colonise the stomach more densely compared to non-virulent strains, resulting in increased levels of inflammation and tissue injury.

The overall aim of this project is to identify molecular mechanism(s) by which virulent *H. pylori* strains subvert the host innate immune surveillance system, allowing for its increased, persistent colonisation in the gastric mucosa.

The specific aims of this project are:

1. To determine the potential role of bacterial virulence factors in modulating human β-defensin gene expression during *H. pylori* infection.

2. To delineate the signal transduction events involved in the regulation of human β-defensin during *H. pylori* infection

CHAPTER 2

Materials and Methods

2.0 Reagents

All cell culture reagents were purchased from Invitrogen, Paisley, UK, unless otherwise stated. Recombinant IL-1β, TNFα, Epidermal growth factor (EGF) and IL-1 receptor antagonist (IL-1RA) were obtained from Peprotech, London, UK. The MAP Kinase inhibitors, SB203580, PD98059, U0126 and SP600125, the NF-κB proteasome inhibitor, MG132, EGFR inhibitors, AG1478 and PD168393, and JAK inhibitor I were from Calbiochem, Nottingham, UK. Non phospho- and phospho-specific antibodies for MAP Kinases (p44/42, p38, SAPK/JNK), phospho-NF-κB p65 (Ser 536), NF-κB inhibitory protein (IκBα and IκBβ) antibodies were attained from Cell Signalling Technology, Hitchin, Hertfordshire, UK and Autogen Bioclear, Wilts, UK respectively. Neutralising EGFR and human β-defensin 2 antibodies were from Autogen Bioclear, Wilts, UK and human β-defensin 3 antibody was from Gentaur Molecular Products, Brussels, Belgium. β-actin antibody was from Sigma, Poole, UK.

2.1 Human epithelial cell-lines

The human gastric epithelial cell line AGS was used throughout the study. This cell-line was maintained in RPMI 1640 culture medium with 2mM L-glutamine supplemented with 10% heat inactivated fetal calf serum (FCS; Sigma, Poole, UK), 100 units/ml penicillin, 100μg/ml streptomycin, and 1% non-essential amino acids. This media is referred to as complete media hereafter.

Human embryonic kidney cell-line (HEK 293) used for transfection experiments was grown in Minimal Essential media (MEM) with 10% heat inactivated FCS, 2mM L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin, 1% non-essential amino acids, and 20mM Hepes buffer (pH 7.2-7.5).

Stably transfected HEK293 cells expressing conditional kinase ΔMEKK3:ER* (HM3 cells) (Todd *et al.*, 2004) and conditional ΔRaf-1:ER* (HR1 cells; kindly provided by Dr Simon Cook, Babraham Institute, Cambridge, UK) were maintained in phenol red-free DMEM high-glucose medium containing 100μg/ml penicillin-streptomycin, 2mM L-glutamine, 10% FCS, 400 μg/ml geneticin or 2μg/ml puromycin (Sigma, Poole, UK). The cells were kept at 37°C and 5% CO_2 in a humidified incubator (Galaxy CO_2 Incubator, Wolf Laboratories, UK).

2.1.1 Cell Passage

Confluent cell-line monolayers were routinely passaged by trypsinisation when appropriate. Cells were washed twice with sterile phosphate buffered saline (PBS) before adding 1x Trypsin-EDTA to cover the cell surface for 5 minutes at 37°C. This allowed adherent cells to detach and trypsin activity was immediately inhibited by the addition of complete media containing 10% FCS. The resultant cell suspension was centrifuged (1000rpm, 10 min, 4°C) (Rotina 46R centrifuge, Wolf Laboratories, UK) followed by resuspension of the cell pellet in complete media prior to centrifugation to minimise any remaining trypsin activity. Cells were seeded and maintained in $25cm^2$ or $75cm^2$ culture flasks, 6 or 96 well plates (Corning Life Sciences, UK) at a concentration as required.

2.1.2 Counting viable cells

10μl of the cell suspension was added to 10μl of 0.4% trypan blue (Sigma, Poole, UK) for 1min. 10μl of this mixture was placed onto a haemocytometer counting chamber (BDH Laboratories, Poole, UK), and live cells counted under a microscope (Zeiss, Germany) at a magnification of x 40. The unstained viable cell count was:

The number of cells within a 25 box field x dilution factor = Total number of cells x 10^4 /ml

2.1.3 Freezing cells

For long term storage, confluent adherent cell-lines were washed twice with sterile PBS and trypsinised as described above. AGS cells were centrifuged (1000rpm, 10min, 4°C) and resuspended in 80% FCS, 10% complete media and 10% sterile dimethyl sulphoxide (DMSO; Sigma, Poole, UK). HEK 293 cells were resuspended in 90% FCS and 10% sterile glycerol (Sigma, Poole, UK). 1ml cell suspension aliquots were transferred into cryovials (Nunc, Roskilde, Denmark) and slowly chilled to and stored at -80°C or liquid nitrogen (New Brunswick Scientific, Jencons PLC, UK).

2.1.4 Thawing cells from -80°C storage

Cryovials were removed from storage and placed immediately into a water bath at 37°C to permit rapid thawing. Thawed cells were added to complete media previously warmed to 37°C and centrifuged (1000rpm, 10min, 4°C) to remove DMSO/ glycerol. The cell pellet was resuspended in complete media, before proceeding with culturing in a 25cm^2 tissue culture flask.

2.2 Bacterial culture

2.2.1 Preparation of blood agar plates

Bacterial reagents for maintaining *Helicobacter pylori* strains including horse blood, agar and selective supplements were purchased from Oxoid, Basingstoke, UK.

Wild type *Helicobacter pylori* strains were grown on blood agar plates prepared using autoclaved Columbia Agar Base, supplemented with 5% horse blood and *Helicobacter pylori* selective (Dent) supplement. *H. pylori* selective supplements comprised: Vancomycin (5mg); Cefsulodin (2.5mg); Trimethoprim lactate (2.5mg) and Amphotericin B (2.5mg). Aseptically 2ml of sterile PBS was added to one vial of selective supplement

and mixed gently to dissolve. The contents were then added to 500ml of Columbia Blood Agar Base cooled to 50-55°C and mixed well before pouring into sterile Petri dishes.

2.2.2 *Helicobacter pylori* strains

Helicobacter pylori strains used throughout the study were kind gifts from Professor John Atherton (Institute of Infections, Immunity and Inflammation, Queens Medical Centre, Nottingham, UK) and Dr Andrew Harris (Institute for Cell and Molecular Sciences, Queen Mary College, London, UK) and stored in glycerol at -80°C (Table 2.1). Bacteria were grown on blood agar plates and maintained under microaerophilic conditions with CampyGen sachets, in aerojars incubated at 37°C. A sterile bacterial loop was utilised to streak bacteria onto a blood agar plate. Isogenic mutants were grown in the presence of kanamycin (50μg/ml; Sigma, Poole, UK) on blood agar plates.

2.3 Co-culture studies

2.3.1 Gastric epithelial cells with *Helicobacter pylori*

After media removal, AGS cells were washed twice with PBS and incubated overnight in low (0.5%) serum media prior to addition of bacteria. Cells were routinely maintained in 0.5% FCS media without antibiotics during bacterial-epithelial co-culture experiments. A multiplicity of infection (MOI) of 100 [i.e., $1x10^6$ epithelial cells/ $1x10^8$ bacteria] was employed (Bajaj-Elliott *et al*, 2002). Bacteria were counted firstly by resuspending colonies in PBS using sterile techniques and quantified by measuring optical density at 450nm (6300 Spectrophotometer, Jenway, UK).

Helicobacter pylori strain	Characterisation
HP 60190	Cytotoxic Wild Type v*acA* s1m1, *cag* positive
HP 60190 :: *cag A*	Isogenic mutant, *cagA* deleted
HP 60190 :: *cag E*	Isogenic mutant, *cagE* deleted
HP 60190 :: *vacA*	Isogenic mutant, *vacA* deleted
HP 84183	Cytotoxic Wild Type *vacA* s1m1, *cag* positive
HP 84183 :: c*ag A*	Isogenic mutant, *cagA* deleted
HP 84183 :: *cag E*	Isogenic mutant, *cagE* deleted
HP 84183 :: *vac A*	Isogenic mutant, *vacA* deleted
HP 26695	Cytotoxic Wild Type
HP J150	Non-cytotoxic strain, *vacA* s2m2, *cag* positive
HP J99	Cytotoxic Genome strain *vacA*, s1m1, *cag* positive
HP Tx30a	Clinical isolate, *cag* PAI negative

Table 2.1 *Helicobacter pylori* strains, clinical isolates and isogenic mutants utilised in the present study.

Bacterial counts were obtained as follows:

OD at 450nm x dilution factor = 1 x 10^8 colony forming units per ml (cfu/ml).

The bacterial suspension was appropriately diluted to a final concentration of 1x10^8/ml and added directly to epithelial cell cultures. Visualisation of cell vacuolation 6-8h post-infection was an indicator of infection.

2.3.2 Epithelial cell stimulation

Cytokine and 4-Hydroxytamoxifen (4-HT; Sigma, Poole, UK) stimulation studies were undertaken in low serum media in the presence of antibiotics. All recombinant cytokines and agonists were reconstituted according to manufacturer's instructions. The following concentrations were used in the present study: Interleukin-1β (IL-1β) 20ng/ml; Tumour necrosis factor- α (TNF-α) 20ng/ml; Interferon-γ (IFN-γ) 40ng/ml (approximately 100U/ml); Interleukin-1 receptor antagonist (IL-1RA) 200ng/ml; 4-Hydroxytamoxifen (4-HT) 100 nM; Recombinant Epidermal Growth Factor (EGF) 50ng/ml. The concentrations utilised are known to have minimal cytotoxic effects on epithelial cells.

2.3.3 Inhibitor studies

All inhibitors were reconstituted and prepared to the relevant stock concentrations according to the manufacturer's instructions. The concentration of inhibitors for specific signal transduction pathways were as follows: p38 inhibitor SB203580, 25μM; ERK inhibitor PD98059, 25μM; ERK inhibitor U0126, 25μM; JNK inhibitor SP100625, 50μM; NF-κB inhibitor MG132, 25μM; EGFR inhibitor AG1478, 3μM; EGFR inhibitor PD168393, 2μM; JAK Inhibitor I, 540nM; EGFR neutralising antibody, 10μg/ml. Routinely, cells were pretreated with inhibitors for 30-60min prior to proceeding with cytokine/ bacterial co-stimulation studies. The presence of the inhibitors was maintained throughout the course of each experiment.

2.4 Gene expression of host defence genes by Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

2.4.1 Total RNA isolation

Reagents for RNA extraction, RT and PCR analyses were from Invitrogen, Paisley, UK unless otherwise stated. Cells were washed twice with PBS and stored at -80°C until required. Total RNA isolation was carried out using a monophasic solution of phenol and guanidine thiocyanate (TRIZOL), of which 1ml was added directly to a 25cm^2 flask or 1 well of a 6 well plate. The cell layer was gently shaken (Rotatest, Denley, UK) for 10min at room temperature to allow complete suspension of cells. The viscous solution (due to cellular DNA) was transferred into a sterile 1.5ml eppendorf. Vigorous repeated dispersion of the solution through a needle and syringe (25gauge, Terumo, Leuven, Belgium) allowed further homogenisation. The resultant Trizol solution was allowed to stand at room temperature for 5min to permit greater dissociation between nucleic acid-protein complexes. 200µl chloroform (Sigma, Poole, UK) was added per ml Trizol used and vortexed thoroughly (Vortex Genie-2, Scientific Industries, NY, USA) for 15sec before centrifugation (13000rpm, 20min, 4°C) (Eppendorf 5415R microfuge, Eppendorf, Cambridge, UK). This procedure allowed organic extraction of the total RNA from the remaining cellular debris. The resultant top aqueous layer (comprising RNA) was aspirated (approximately 500µl) and transferred into a fresh eppendorf. RNA was precipitated by the addition of 500µl isopropanol (Sigma, Poole, UK). Extracted RNA was stored at -80°C.

2.4.2 Spectrophotometric quantification of total RNA

Total RNA was pelleted by centrifugation (13000rpm, 20min, 4°C) and washed with ice-cold 70% ethanol (BDH Laboratories, Poole, UK). The RNA pellet was either vacuum (Heto Vacuum Centrifuge, Jencons PLC, UK) or air-dried to remove traces of ethanol prior

to the addition of RNase- free water (Sigma, Poole, UK). Appropriate amounts of resuspended RNA solution were diluted in water and concentration determined by absorbance (U-1800 Spectrophotometer, Digilab Hitachi, Tokyo, Japan) at 260nm and 280nm. The amount of RNA (μg/μl) was calculated as follows: [(reading at 260nm) x (dilution factor) x 40] / 1000.

Typically, 1 x 10^6 cells would yield approximately 20-25μg.

2.4.3 Reverse transcription (RT)

5μg of total cellular RNA was transcribed to complementary DNA (cDNA) using Moloney murine leukaemia virus reverse transcriptase. In a typical RT reaction, 1μl (0.5μg) oligo-dT was added to 5μg of RNA and heated to 70°C for 10min followed by immediate chilling on ice to permit greater specific annealing between oligo-dT and poly-A tail of mRNA molecules.

The remaining components of the reaction: 5 x First strand buffer, 4μl; 0.1M DTT, 2μl; 10mM deoxynucleotide triphosphates (dNTP) mix, 1μl; 100U Enzyme (reverse transcriptase), 0.5μl; were then added and the reaction allowed to proceed at 42°C for 1h. The reaction was terminated by heat inactivated at 70°C for 10min.

2.4.4 Polymerase Chain Reaction (PCR)

cDNA obtained was used in subsequent PCR reactions. Reaction volumes were 25μl, of which 12.5μl of 2x PCR master mix (Biomix Red, Bioline Ltd, London, UK) encompassed PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs and *Taq* polymerase. In addition, 2μl of 20 pmol of each oligonucleotide primer and 2μl cDNA were included to complete the reaction mix. Primers utilised are listed in Table 2.2. The remainder consisted of RNase free water to total 25μl. Master mixes were prepared for multiple reactions to minimise inter-sample variation. Contents of the PCR reaction (Abgene, Epsom, Surrey, UK) were mixed by brief

centrifugation. PCR amplification was carried out in a thermal cycler (Peltier Thermal Cycler, DNA Engine Systems) as follows:

a) 94°C for 3min to denature the template - 1 cycle

b) 94°C denaturation for 90 sec
58°C annealing for 90 sec
72°C extension for 90 sec
} - 32-37 cycles

c) 72°C for 10min -1 cycle

Finally, PCR products were quantified by agarose gel electrophoresis (Flowgen, Nottingham, UK). 2% agarose (Invitrogen, Life Technologies, Paisley, UK) gels were prepared in 1 x Tris borate EDTA (TBE) buffer (National Diagnostics, UK) and bands visualised by ethidium bromide (Sigma, Poole, UK) staining. Semi-quantitative analyses were conducted by densitometric measurements of bands followed by normalisation to the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Alpha Imager, Multimage Light Cabinet, Alpha Innotech Corporation, Essex, UK).

Target gene	Sense primer	Anti-sense primer	Product size (bp)
hBD1	^{109}TTG TCT GAG ATG GCC TCA GGT GGT AAC^{136}	^{362}ATA CTT CAA AAG CAA TTT TCC TTT AT^{337}	253
hBD2	^{11}CCA GCC ATC AGC CAT GAG GGT CTT^{34}	^{287}CAT GTC GCA CGT CTC TGA TGA GGG AGC^{261}	276
hBD3	^{202}GGT GAA GCC TAG CAG CTA TGA GGA TC^{227}	^{385}GAG CAC TTG CCG ATC TGT TCC TCC^{362}	183
IL-8	^{41}ATG ACT TCC AAG CTG GCC GTG^{62}	^{332}TCT CAG CCC TCT TCA AAA ACT TCT C^{308}	291
NOD1	^{634}ATT CTG GAC CTG GTA CAG AGC^{655}	^{998}CAG GAT GAA GAT GGT CTC ACC^{977}	364

COX2	^{477}CCT TCT CTA ACC TCT CCT ATT ATA CTA G^{504}	^{878}AGA TCA TCT CTG CCT GAG TAT CTT^{855}	401
IL-18	^{119}GCT GCT GAA CCA GTA GAA GAC AAT TGC^{225}	^{583}CCT TGATGT TAT CAG GAGGAT TC^{561}	464
Lysozyme	^{108}GAA CTC TGA AAA GAT TGG GAA TGG A^{132}	^{463}ACA ACC TTG AAC ATA CTG ACG GAC A^{439}	355
GAPDH	^{630}CTA CTG GCG CTG GCA AGG CTG T^{651}	^{989}GCC ATG AGG TCC ACC ACC CTG CTG^{966}	358

Table 2.2 Sequences of synthetic oligonucleotide primers used to study innate host defence gene expression.

2.5 Western Blotting

2.5.1 Detection of signal transduction components

Equipment and reagents for Western blotting were from Bio-Rad Laboratories, Hemel Hempstead, UK and Amersham Biosciences, St Albans, UK. All buffer recipes are detailed in Table 2.3. Experiments were routinely set up in 25cm^2 flasks or 6 well tissue culture plates, at the end of which media was aspirated, cells washed twice with PBS prior to lysis in 250µl 2x Laemmli sample buffer. Cell lysates were agitated on a rotary shaker (Rotatest shaker, Denley, UK) to obtain a cell suspension which was transferred into 1.5ml eppendorf, and homogenised by shearing and sonication (Bandelin Sonopuls, Bandelin Electronics, Berlin, Germany) for 10-15sec on a power setting of approximately 60%. Samples were boiled at 95-100°C for 5min and cooled on ice before being micro-centrifuged for a further 5min. Supernatants were then subjected to 7-10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-Protean 3 cell unit (Sambrook and Russell, 2001). 5µl of ColorBurst prestained Electrophoresis markers (Sigma, Poole, UK) was run for molecular weight determination and a visual check for transfer efficiency.

Typically, 10µl of sample was loaded into each well and run at a voltage of 100V for an appropriate length of time. Proteins were transferred onto nitrocellulose membrane by semi-dry blotting (12V for 45min) using Transfer blot, Semi-dry transfer cell. Prior to transfer, blotting paper (VWR, Lutterworth, UK), nitrocellulose membrane (Hybond ECL) and the SDS-PAGE gel were allowed to equilibrate in chilled transfer buffer for at least 5min. After transfer non-specific binding was blocked by incubating the membrane in blocking buffer for 1-2h at room temperature with gentle shaking. This was followed by overnight incubation with phospho-specific primary antibody (see Table 2.4) in blocking buffer on a suspension mixer at 4°C.

	Tris-Glycine	**Tris-Tricine**
SDS Sample buffer	(4x) 1ml Tris (0.5M, pH 6.8), 0.8ml Glycerol, 1.6ml 10% SDS, 0.4ml β-mercaptoethanol, 0.2% Bromophenol blue, 3.8ml Deionised water	(1x) 200mM Tris-HCl (pH 6.8), 40% glycerol, 2% SDS, 0.04% Coomassie Blue
Electrophoresis Buffer pH 8.3	25mM Tris, 192mM Glycine, 0.1% SDS	100mM Tris, 100mM Tricine, 0.1% SDS
Transfer Buffer	25 mM Tris, 0.2 M Glycine, 20% Methanol	100mM Tris, 100mM Tricine
Blocking buffer	1 x TBS/0.1 % Tween-20, 5% w/v nonfat dry milk (Marvel)	1 x TBS/0.1 % Tween-20, 5% w/v nonfat dry milk (Marvel)

Table 2.3 Buffer composition for Tris-Glycine and Tris-Tricine SDS PAGE. All reagents were purchased from Sigma or BDH Laboratories, Poole, UK.

Primary Antibody	Source	Dilution	Company
Phospho p44/42 MAP Kinase (Thr202/Tyr204)	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
P44/42 MAP Kinase	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
Phospho p38 Map Kinase (Thr180/Tyr182)	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
P38 Map Kinase	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
Phospho SAPK/JNK Map Kinase (Thr183/Tyr185)	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
SAPK/JNK Map Kinase	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
Phospho NF-κB p65 (Ser536)	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
NF-κB p65	Goat polyclonal IgG	1:250	Santa Cruz Biotechnology
IκB-α	Rabbit polyclonal IgG	1:1000	Cell Signalling Technology
IκBβ	Rabbit polyclonal IgG	1:250	Santa Cruz Biotechnology
β-actin	Mouse monoclonal IgG1	1:2000	Sigma
hBD2	Goat polyclonal IgG	1:500	Autogen Bioclear
hBD3	Rabbit polyclonal IgG	1:1000	Gentaur Molecular Products
Phospho-tyrosine	Mouse polyclonal IgG	1:10000	
Epidermal Growth Factor	Mouse polyclonal IgG	1:1000	
Secondary Antibody			
Anti-mouse antibody conjugated to horseradish peroxidase	Sheep anti-mouse IgG	1:2000	Amersham Biosciences
Anti-rabbit antibody conjugated to horseradish peroxidase	Goat anti-rabbit IgG	1:2000	Cell Signalling Technology
Anti-goat antibody conjugated to horseradish peroxidase	Rabbit anti-goat IgG	1:2000	Dakocytomation Ltd

Table 2.4 Antibodies utilised in the present study.

After 3-4 washes in Tris- buffered saline with 0.1% Tween-20 (TBS/T; Sigma, Poole, UK) for 10min each, the blot was incubated in relevant secondary antibody for 1h on the rotary shaker at RT. Blots were washed 3-4 times for 10min with TBS/T before being exposed to an enhanced chemiluminescence (ECL) reaction for 5min, where 1ml of reagent A was added to 25µl of reagent B (ECL plus, Amersham Biosciences, UK). The excess solution was drained and membrane placed between a sheet of clingfilm prior to development. The membrane was exposed to an x-ray film (Hyperfilm ECL) ranging from 30sec to 15min and developed. To ensure equal protein loading, the same membrane was stripped in 1x stripping solution (Re-blot plus mild solution, Chemicon, UK) for 20min, blocked in blocking buffer, for 1-2h with gentle agitation and re-probed with a monoclonal anti-β-actin antibody or the corresponding non-phospho specific antibody.

2.5.2 Human β-defensin Peptide detection

Both uninfected control, infected supernatants and cell lysates were subjected to detection for β-defensin peptides; hBD2 and hBD3. Protein was quantified by BCA assay (Sigma, Poole, UK) as described below. Tris-Tricine loading buffer (Bio-Rad Laboratories, Hemel Hampstead, UK) was added directly to 150µg of total protein prior to the mixture being subjected to 16% Tris-Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Table 2.5). Tris-tricine markers (Bio-Rad Laboratories, Hemel Hempstead, UK) were loaded alongside samples, as were recombinant peptides (hBD2 is 4.3kDa and hBD3 is 5.1kDa). After electrophoresis, gels were transferred onto a polyvinylidene difluoride (PVDF) membrane at 0.8mA/cm^2 for 40min. The membrane was exposed to UV cross-linking (auto-crosslink setting) followed by blocking in 5% non-fat milk/TBS for 1h. The blots were incubated in 1:500 dilution for hBD2 and 1:1,000 for hBD3 overnight, followed by three washes with TBST prior to incubation with horseradish peroxidase-

conjugated rabbit anti-goat IgG and goat anti-rabbit IgG (1:1,000; Dako Ltd., High Wycombe, UK), respectively. The reaction was developed by ECL as stated above.

Components for 16% Resolving gel	**Component Volume (~30ml) 1 large gel**
Water	5.97
Glycerol	4g (≅ 3.17ml)
30% Acrylamide mix (Protogel, National Diagnostics, UK)	10.86
3 M Tris-Cl (pH 8.45)	10.0
10% SDS	0.3
10% Ammonium persulphate	0.05
TEMED	0.015
5% Stacking gel	**Component Volume (~20ml) for 2 gels**
Water	10.0
30% Acrylamide mix	3.4
3 M Tris-Cl (pH 8.45)	6.2
10% SDS	0.2
10% Ammonium persulphate	0.05
TEMED	0.025

Table 2.5 Composition of Tris-Tricine SDS- Polyacrylamide Gels.

2.5.3 Protein Determination

Protein levels were determined using a Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma, Poole, UK). The principle is similar to the Lowry procedure; both rely on the formation of a Cu^{2+} protein complex under alkaline conditions, followed by reduction of

Cu^{2+} to Cu^{1+} which is monitored by the production of a purple-blue complex (Smith, 1985). The amount of reduction is proportionate to protein concentration. The BCA working reagent was prepared by mixing 50 parts of Reagent A (Bicinchoninic acid) with 1 part of Reagent B (Copper sulphate). 20 parts of the BCA working reagent was then mixed with 1 part of protein sample. A blank with buffer alone, Bovine Serum Albumin (BSA) standards of varying concentrations (2μg to 25μg) were also included. BCA was added to each protein sample, vortexed gently and left at 37°C for 30min. The solutions were transferred into cuvettes and OD readings taken at 562nm. Protein concentration in samples was determined using the standard curve plotted for BSA.

2.6 Transformation of Plasmid DNA into Competent *E.coli* cells

Renilla, IL-8, A46R and A52R plasmids were generous gifts from Dr Andrew Bowie (Trinity College, Dublin, Ireland). NOD1 and NOD2 siRNA plasmids were kindly provided by Dr Barbara Manzo (Queen Mary School of Medicine and Dentistry, London, UK). Luciferase reporter constructs of hBD2 and hBD3 were kind gifts from Mr Shao-ren Wang (Institute of Child Health, London, UK) and Dr Ole Sorensen (Lund University, Lund, Sweden) respectively.

Plasmid DNA received was first transformed in JM109 competent cells (Promega, Southampton, UK). The procedure involved thawing out competent cells on ice and DNA (1-10ng) was added by gentle flicking to 50μl of thawed cells and left for 30min on ice. Cells/DNA complex were heat-shocked for 2min at 42°C and immediately placed on ice for a further 5min. 1ml LB (Luria Bertani) broth (Merck, Hoddesdon, Hertfordshire, UK) containing 10mM Mg^{2+} and 10mM D-glucose was added to each transformation reaction, which was further incubated for an hour at 37°C with gentle shaking. Ampicillin (100μg/ml) plates were prepared with LB agar and various dilutions of the transformation

reaction were plated and incubated overnight at 37°C. The following day colonies were appropriately analysed.

2.7 Purification of Plasmid DNA

Plasmid DNA was purified using Qiagen Maxi-prep kits based on a modified alkaline lysis procedure, according to the manufacturer's instructions (Crawley, West Sussex, UK). Briefly, a single colony was used to inoculate a starter culture of 5ml LB broth (ampicillin 100μg/ml) in a sterile 50ml falcon tube and incubated for a few hours at 37°C with vigorous shaking (300rpm). The starter culture was diluted further (1 in 50) and shaken overnight at 37°C for a large scale plasmid preparation.

Bacterial cells were harvested 16h later by centrifugation (6000rpm, 15min, 4°C) (Sorvall GSA rotor, Hertfordshire, UK). Bacterial pellet was resuspended in 10ml Buffer P1, Buffer P2 was added and mixed by inversion several times and incubated at room temperature for 5min. 10ml chilled Buffer P3 was then added and mixed by inversion before being left on ice for 20min. The latter step allows precipitation of proteins and chromosomal DNA from the lysed bacterial cell suspension.

The precipitated cellular debris was removed by centrifugation (13,000rpm, 30min, 4°C). The resultant supernatant containing plasmid DNA was removed and centrifugation repeated for 15min. The supernatant was further purified using the Qiagen-tip column. The column was equilibrated in Buffer QBT prior to usage. Once the supernatant was applied, the column was washed twice with 30ml Buffer QC following elution of the plasmid DNA with 15ml Buffer QF. Plasmid DNA was precipitated by the addition of 0.7 volumes isopropanol and centrifuged promptly at 13,000rpm for 10min. The resultant DNA pellet was allowed to air-dry for 10min before the addition of TE buffer, pH 8. An aliquot was run on a 1% agarose gel for determination of yield and purity which was verified by

spectrophotometry: DNA (μg/μl) was calculated as follows: [(reading at 260nm) x (dilution factor) x 50] / 1000). Plasmid DNA was stored in aliquots at -20°C until required or stored long term as bacterial glycerol stocks.

2.8 Transient transfection of epithelial cell-lines

HEK 293 and AGS cells were plated in a 96-well plate at a density of 2 x 10^4 cells /well in 200μl complete media. Transfections were conducted the following day using FuGene reagent (Roche, Lewes, UK) as recommended. All experiments were performed at a cell confluency of 50-80% with FuGene transfection reagent (μl) to DNA (μg) ratio of 4:1. Each transfection comprised a total DNA content of 230ng. This included:

a) Test plasmid i.e. Firefly luciferase constructs under investigation, IL-8, hBD2 or hBD3 promoter.
b) HSV TK *Renilla* luciferase construct, to correct for transfection efficiency
c) Empty vector (pcDNA3.1; Stratagene, Cambridge, UK) to make up the appropriate amount of DNA for each experiment.
d) Additional signalling pathway plasmids A46R, A52R (Vaccinia Virus proteins), siNOD1 or siNOD2.

20ng of *Renilla* control plasmid was co-transfected with each experimental plasmid (60ng) and 150ng of empty vector was also included to give the final concentration of 230ng. Each experimental condition was performed in triplicate (Bowie *et al.*, 2000).

A master mix of transfection reagent in Optimem low serum media (Invitrogen, Paisley, UK) was prepared. 0.8μl of FuGene was added to 9.2μl Optimem low for each individual well. A negative control (empty vector alone and no DNA) was also included. The DNA mix and FuGene/Optimem were incubated for a minimum of 20min at room temperature to

allow DNA-FuGene complex formation, before being added to cells. Transfections were allowed to proceed for 24h prior to cytokine or bacterial stimulation. At the end of each experiment, cells were harvested by removing media followed by washing with ice-cold PBS. 50µl of 1 x passive lysis buffer (Promega, Southampton, UK) was added to each well. Plates were stored at -20°C until analysis, at which stage 25µl cell lysate was transferred into 2 white luciferase assay plates (Labtech International, Ringmer, East Sussex, UK). One plate was used for the analysis of Renilla luciferase activity, where 40µl of its substrate, Coelentrazine (2µg/ml; Insight Technologies, London, UK) was added to each well. 100µl firefly luciferase substrate (Promega, Southampton, UK) was added to each well in the other plate for the detection of luciferase activation. IL-1β (20ng/ml), a potent agonist for IL-8 and hBD2 gene expression was included as a positive control in all experiments.

Reporter gene activity was quantified using a 96 well plate luminometer (LUCY1 luminometer, Anthos Labtech Instruments, Austria). All experiments were conducted at least twice and performed in triplicate. Luciferase activities were plotted as fold induction compared to activity measured in unstimulated control cells.

2.9 Short Interfering RNA (siRNA) experiments

Plasmids encoding NOD1 and NOD2 (Dr Barbara Manzo, Queen Mary School of Medicine and Dentistry, London) or commercially available double-stranded NOD1 siRNA (Cat #: 16708; Ambion Europe Ltd, Huntingdon, UK) were utilised in this series of experiments.

NOD1 or NOD2 siRNA plasmid (100ng/well) was introduced to cells with empty vector pcDNA3.1 (total DNA concentration 230ng/well) using FuGene transfection reagent 24h prior to transfection with test plasmids as described above.

A second strategy targeting endogenous NOD1 (sequence; sense: GGC CAA AGU CUA UGA AGA Utt anti-sense: AUC UUC AUA GAC UUU GGC Ctc) was carried out utilising

a reverse transfection method. Cells were trypsinised, washed and resuspended to a concentration of 1 x 10^4 cells/ml. 25nM and 50nM commercially available dsRNA NOD1 siRNA was added to Optimem media (10µl/well). Transfection reagent specific for reverse transfection, siPort NeoFX^{TM}, (0.5µl/well; Ambion Europe Ltd, Huntingdon, UK) was added to Optimem media (10µl/well) and combined to the siRNA-Optimem mix. The mixture was left for 20min at room temperature and 20µl added to each well of a 96 well culture plate followed by addition of 80µl cell suspension. Media was replaced 24h after transfection and β-defensin promoter constructs were introduced *via* FuGene transfection method as described above 48h after initial transfection of siRNA. All subsequent steps were carried out as described for transfection of luciferase- promoter constructs.

2.10 Statistics

Results are presented as means ± SEM of three experiments each performed in triplicate. Statistical analyses were performed using SPSS and GraphPad Instat statistical software, variables were compared using a T-test and a probability value of less than 0.05 was regarded as significant.

CHAPTER 3

Role of *H. pylori* Virulence Factors in mediating β-defensin Expression

3.0 Background

Epithelial cells represent a first line of defence between host and the environment. Not only does the epithelium protect by presenting a physical barrier against pathogens but also through mounting innate immune response(s) including the induction of chemokines (e.g. IL-8) and antimicrobial peptides such as β-defensins (Bajaj-Elliott *et al.*, 2002, Crabtree *et al.*, 1993, Keates *et al.*, 1997, O'Neil *et al.*, 2000). Most studies to date have been confined to studying effect of cytotoxic *H. pylori* strains on hBD2 expression (Bajaj-Elliott *et al.*, 2002, George *et al.*, 2003, Hamanaka *et al.*, 2001, O'Neil *et al.*, 2000, Wada *et al.*, 1999, Wada *et al.*, 2001), although Wada and colleagues looked at the contribution of *Cag*PAI, at present, the potential role of bacterial virulence factors in mediating β-defensin expression is unknown.

In order to address the potential contribution of *H. pylori* virulence determinants in mediating β-defensin expression, several isogenic mutant strains including *cagA*-ve strain (lacking the bacterial effector protein), *cagE*-ve strain (lacking a component of the type IV secretion system rendering it dysfunctional), *vacA*-ve strain (lacking the secreted exotoxin which causes vacuolation of host cells) and finally *cag*PAI-ve strain (deficient in the whole syringe system) were utilised.

3.1 β-defensin expression in response to *H. pylori*

Firstly the effect of epithelial cell infection with wild type cytotoxic *H. pylori* strains (*vacA*+ve and *cag*+ve) was assessed. hBD1 expression was found in control uninfected cells and this constitutive expression was observed throughout the infection (Figure 3.1; lanes A, D and E). In contrast, the expression of two other members of the family hBD2 and -3 was inducible (Figure 3.1; lane D, E). Although, hBD2 has been demonstrated by

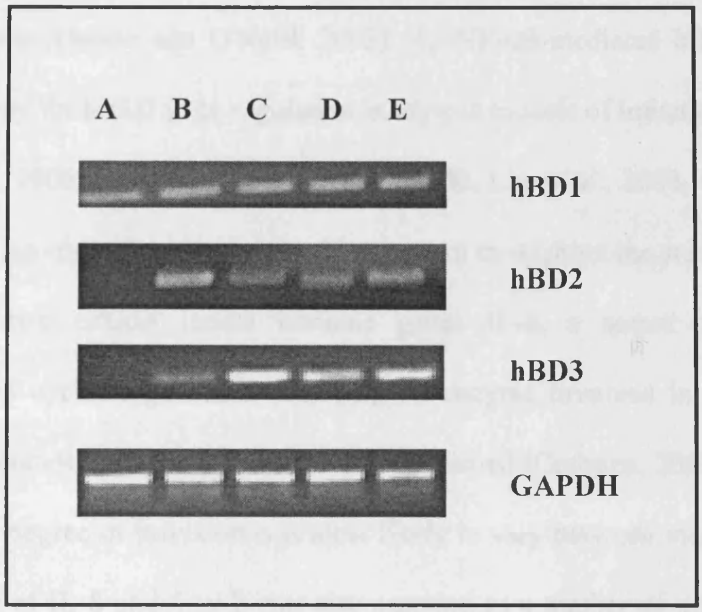


Figure 3.1: Expression of β- defensins during *H. pylori* infection. hBD1 mRNA is constitutively expressed by gastric epithelial cells, whereas hBD2 and 3 are both induced by IL-1β and *H. pylori* 8h post-stimulation. Lane A shows control unstimulated cells, Lane B and C represent cells stimulated with IL-1β and TNFα (both at 20ng/ml concentration) respectively. Cells also expressed β-defensins upon treatment with two cytotoxic wild type *H. pylori* strains ($1x10^8$cfu/ml *Hp 60190, 84-183*) as seen in Lane D and E. Results shown are representative data from three experiments.

several studies, this is the first study showing induction of hBD3 gene expression by *H. pylori,* this data has now been reported (George *et al.*, 2003). Binding of IL-1β to its receptor is known to trigger signal transduction events resulting in activation of NF-κB in many cell types (Dunne and O'Neill, 2003). As NF-κB-mediated hBD2 expression is a defined pathway for hBD2 gene regulation in various models of infection and inflammation (Harder *et al.*, 2000, Krisanaprakornkit *et al.*, 2000, Liu *et al.*, 2003, O'Neil *et al.*, 1999), IL-1β was the agonist of choice as a positive control throughout the study.

The role of two critical innate immune genes IL-8, a potent neutrophil-activating chemokine and cyclooxygenase-2 (COX-2), an enzyme involved in the pathogenesis of gastric inflammation and carcinoma, is well established (Crabtree, 2001, Juttner, 2003). As the extent and degree of infection was most likely to vary between individual experiments, the expression of IL-8 and Cox-2 was also assessed as a marker of infection. The kinetics of IL-8, Cox-2 and β-defensin gene expression was followed by time-course studies both during bacterial infection and cytokine stimulation (Figure 3.2). Expression of hBD2 was rapid with maximal induction observed 6h post-infection (Figure 3.2a; uppermost panel) and declined slowly thereafter. Similar kinetics of hBD2 gene expression were also observed in the presence of IL-1β (Figure 3.2b; uppermost panel). In contrast to hBD2, induction of hBD3 was slow, reaching maximal levels between 12-24h (Figure 3.2; second panel). Interestingly, hBD2 expression was consistently more potently induced in response to IL-1β stimulation compared to *H. pylori*, whereas there was not such a difference noted for IL-8 or hBD3 expression exposed to the different stimuli. Both IL-8 and Cox-2 expression was found to be constitutive in unstimulated AGS cells and further induction was observed in particular when exposed to wild type *H. pylori* (Figure 3.2a; third and fourth panels). Induction of the genes by 6hrs suggests that these innate genes are early response genes and it is likely that shared signal transduction events are involved in the

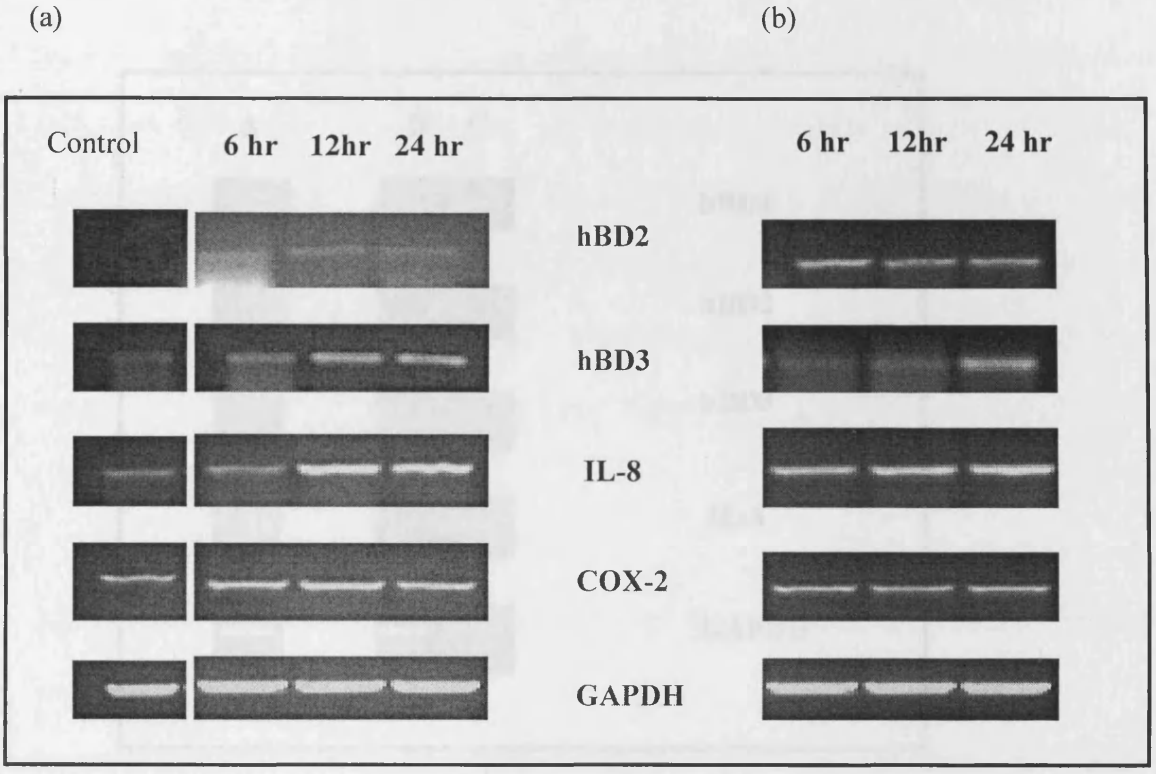


Figure 3.2 Time course of induction of host defence genes by (a) *Helicobacter pylori* and (b) IL-1β. Confluent AGS cells were infected with (a) wild type *H. pylori* strain 26695 ($1x10^8$cfu/ml) or (b) IL-1β. Gene expression was compared to levels in control untreated cells for each gene of interest. Results shown are representative data from three experiments.

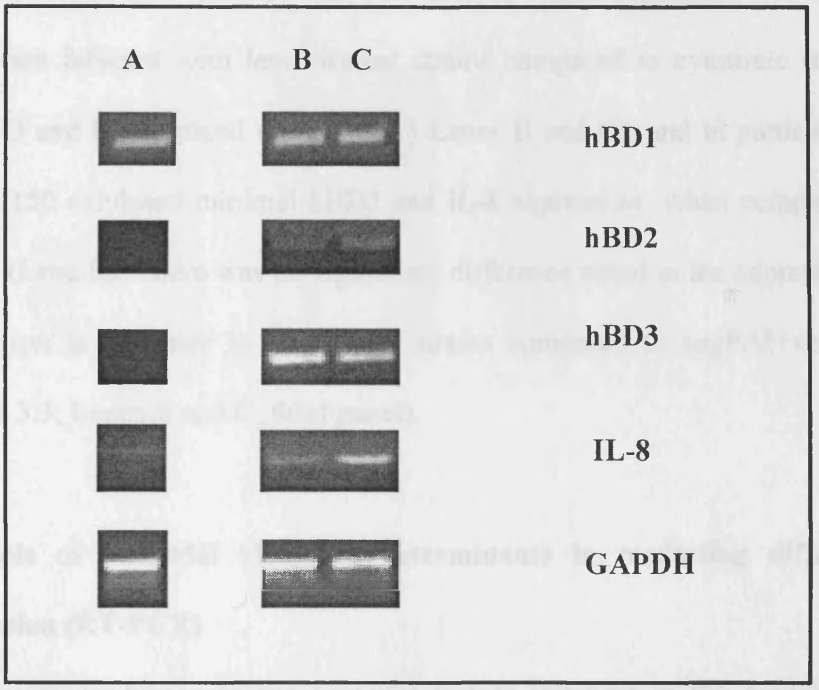


Figure 3.3: Innate immune expression with different clinical *H. pylori* strains. AGS cells were infected with several *H. pylori* strains ($1x10^8$cfu/ml). Results were obtained by RT-PCR analyses. Lane A shows control unstimulated cells, Lanes B and C correspond to cells treated with *H. pylori* strains J150 and J99 respectively.

regulation of their gene expression. To further the above findings the study was expanded beyond the more virulent cytotoxic strains (60190, 84-183, 26695) and investigated the host innate immune response to less virulent strains. J150 (*cag*PAI-ve, s2/m2) and J99 (*cag*PAI+ve, s1/m2 VacA) strains were utilised. hBD2 expression was more modest when cells were infected with less virulent strains compared to cytotoxic strains (Figure 3.1; Lanes D and E compared to Figure 3.3 Lanes B and C), and in particular the *cag*PAI-ve strain J150 exhibited minimal hBD2 and IL-8 expression when compared to *cag*PAI+ve strains (Lane B). There was no significant difference noted in the expression of hBD3 gene expression in response to *cag*PAI-ve strains compared to *cag*PAI+ve *H. pylori* strains (Figure 3.3; Lanes B and C, third panel).

3.2 Role of bacterial virulence determinants in mediating differential β-defensin expression (RT-PCR)

In order to explore potential bacterial factors involved in the modulation of β-defensin regulation, studies utilising isogenic mutants (derived from *H. pylori* 60190) were conducted. The isogenic mutants lacking the bacterial effector protein, CagA-ve and disruption of the type IV secretion apparatus, *cagE*-ve, from parent wild type strains were employed in preliminary experiments to determine differential expression of innate immune genes. Additionally a strain lacking the vacuolating toxin (*vacA-ve*) and one lacking the entire pathogenicity island (*Tx30a*) was included. Bacterial-AGS co-culture studies were performed and data for β-defensin expression obtained 8h post-infection by RT-PCR are shown in Figure 3.4. Although previous studies have suggested a *Cag*PAI requirement for IL-8 production (Crabtree *et al.*, 1999, Fischer *et al.*, 2001, Munzenmaier *et al.*, 1997, Naumann, 2000, Selbach *et al.*, 2002, Viala *et al.*, 2004), this is the first time isogenic mutants have been employed to investigate potential differential hBD expression.

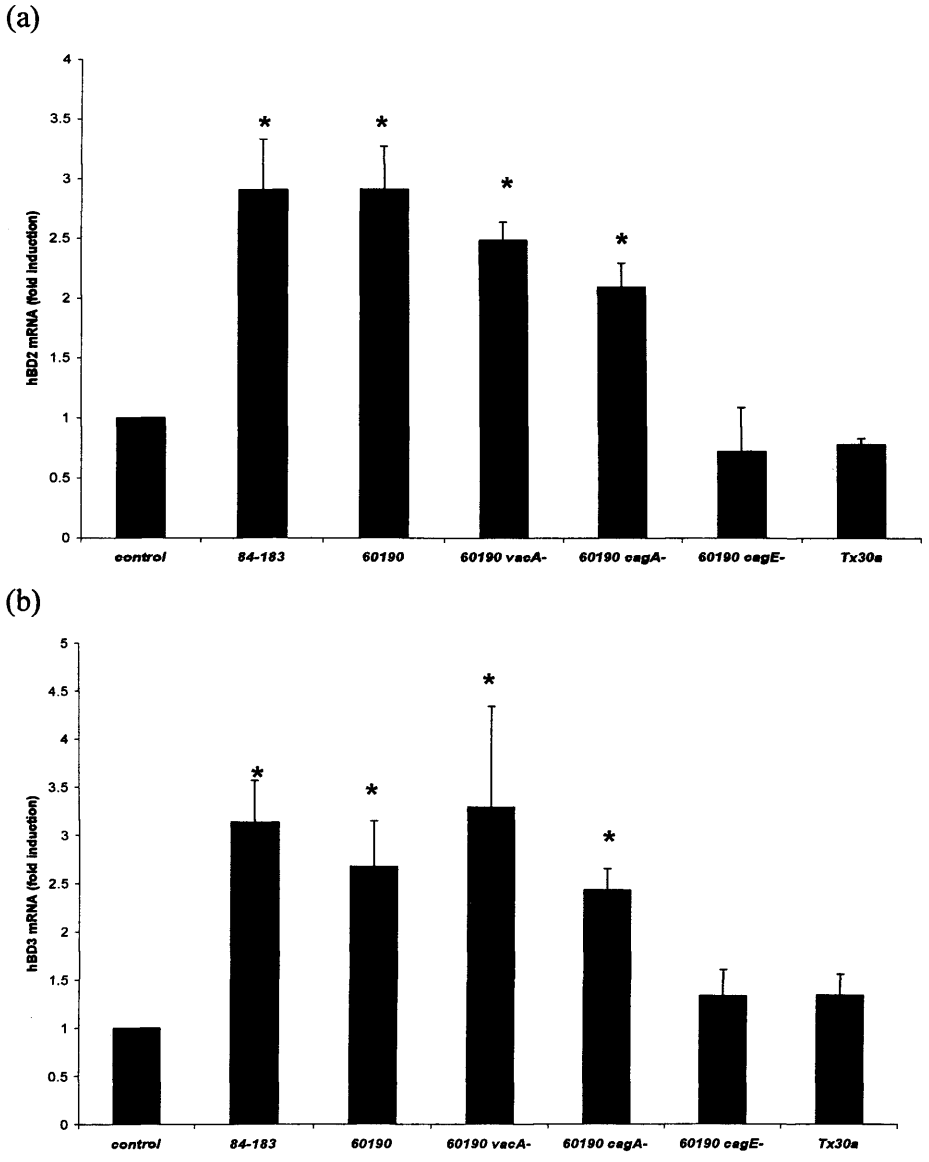


Figure 3.4: Modulation of human β-defensin expression during *H. pylori* infection. AGS cells were co-infected with wild type *H. pylori* strains (60190 and 84-183), isogenic (*vacA*, *cagA*, and *cagE*) mutants of strain 60190,) and a strain lacking the *cag*PAI (Tx30a) (MOI=100). hBD2 **(a)** and hBD3 **(b)** gene expression 8h post-infection was determined and normalised to *GAPDH*. Increases in mRNA levels are expressed as n-fold induction compared to uninfected control cells. Data shown is mean (± SEM; * $p < 0.05$) induction of three independent experiments.

Infection in the presence of cytotoxic strains 84-183 and 60190 caused a significant ($p < 0.05$) increase in hBD2 gene expression, as described earlier. Both the *cagA* and *vacA* mutant strains were able to induce hBD2 gene expression to a similar extent as the wild type parent strains (Figure 3.4a). In contrast, no significant increase in hBD2 gene expression over control was observed during infection with either the 60190 *cagE* mutant or strain Tx30a, suggesting that the type IV secretion system plays a critical role in hBD2 gene expression.

For hBD3, an approximate 2.5- to 3-fold increase ($p < 0.05$) in mRNA expression was noted during infections in the presence of the wild type strains verifying our previous results. As observed for hBD2 gene expression (Figure 3.4a), the absence of the CagA and VacA virulence factors did not affect the degree of hBD3 mRNA induction (Figure 3.4b), suggesting these two bacterial proteins do not play a significant role in *H. pylori*-mediated β-defensin gene expression.

A role for an intact *Cag*PAI in mediating IL-8 gene expression was confirmed, and comparably to hBD2 and -3, there did not seem to be a role for the bacterial CagA in the regulation of IL-8 mRNA expression (Figure 3.5). Interestingly, both IL-18 and COX-2 gene expression was found to be less dependent on the presence of the *Cag*PAI in this series of experiments (Figure 3.5), indicating that these innate immune genes are regulated by *Cag*PAI-independent mechanisms.

3.3 Role of bacterial virulence determinants in mediating differential β-defensin expression (Transfection analyses)

(a) Optimisation studies with IL-8, NF-κB promoters in HEK 293 cells

To confirm and verify the findings from the RT-PCR analyses, transfection experiments with innate defence promoter constructs were conducted. Preliminary studies optimising

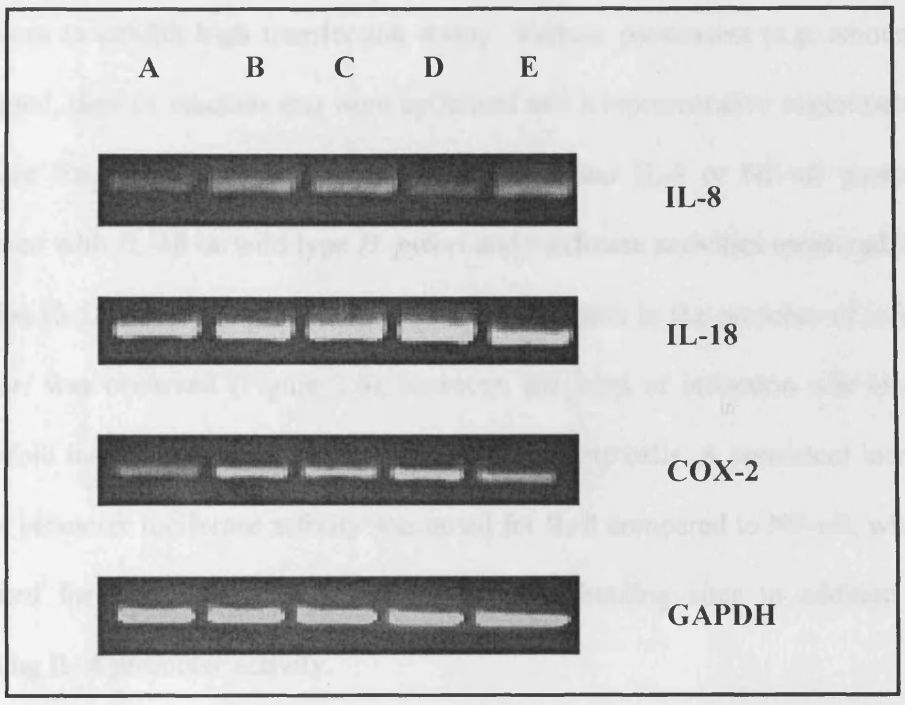


Figure 3.5: Expression of innate immune genes by *H. pylori* isogenic mutants. AGS cells were infected with several Isogenic *H. pylori* mutants ($1x10^8$cfu/ml). RNA from infected gastric epithelial cells was amplified by RT-PCR. Lane A shows control unstimulated cells, Lane B represent cells stimulated with wild type strain 84183. Lane C and D correspond to cells treated with *cagA* negative and *cagE* negative isogenic mutants from parent strain 84183, respectively. A panel of innate immune genes was studied and experiments were carried out three times, a representative result is shown. Lane E was included for a positive control with IL-1β stimulated AGS cells.

experimental conditions were conducted utilising NF-κB and IL-8 promoter constructs in HEK 293 cells. These cells were chosen prior to investigating AGS cells as HEK 293 cells are known to exhibit high transfection ability. Various parameters (e.g. amount of DNA transfected, time of reaction etc) were optimised and a representative experiment is shown in Figure 3.6. HEK 293 cells transfected with either IL-8 or NF-κB promoters were stimulated with IL-1β or wild type *H. pylori* and luciferase activities measured. Significant induction (5-18 fold) of both IL-8 and NF-κB expression in the presence of infection with *H. pylori* was observed (Figure 3.6), however, the level of induction was less dramatic, only 2-fold induction when IL-1β was used to stimulate cells. A consistent increase in the level of promoter luciferase activity was noted for IL-8 compared to NF-κB, which may be accounted for by the various transcription factor binding sites in addition to NF-κB regulating IL-8 promoter activity.

(b) Studies with hBD2 and -3 in AGS cells

The full length (2kB) hBD2 promoter was constructed in our laboratory (Mr Shao Wang, London, UK; personal communication) and the hBD3 promoter was a kind gift from Ole Sorensen (Lund University, Sweden), therefore it was pertinent to firstly ascertain the functional (i.e., inducible promoter activity) of the two reagents. Transfections were conducted as before in HEK 293 cells using 60ng hBD2 promoter plasmid per well. Each experimental condition was carried out in triplicate. hBD2 luciferase promoter construct exhibited significant increased activity upon both bacterial and cytokine stimulation (Figure 3.7). An approximate 5 fold induction was observed. As the optimal conditions of transfection for hBD2 promoter had not been established, studies using another transfection method were carried out by the use of peptide-mediated transfection as an alternative to Fugene transfection reagent (data not shown).

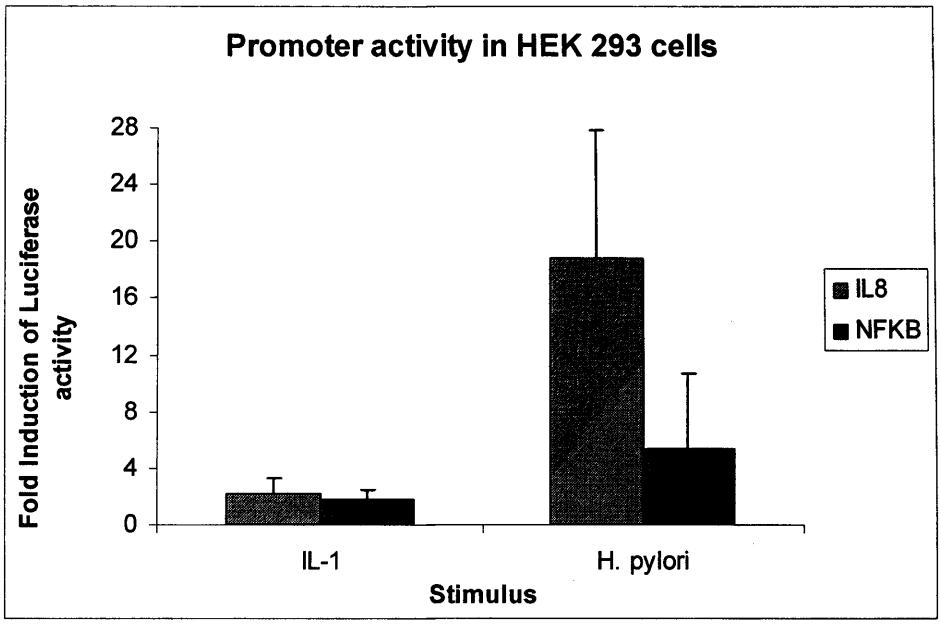


Figure 3.6 *H. pylori* is a potent inducer of IL-8 and NF-κB. HEK 293 cells were transiently co-transfected with pRL-TK-luciferase and pGL3-control vector and either NF-κB or IL-8 promoter luciferase reporter plasmids (total 230ng DNA) and incubated for 24h. IL-1β (20ng/ml) or *H. pylori* (strain 60190 $1x10^8$cfu/ml) were added for 16h before cells were harvested in passive lysis buffer. Promoter luciferase activities were analysed by luciferase reporter assay system and normalised using pRL-TK-luciferase activity (*Renilla* luciferase activity) in each sample. Data represent the mean (fold induction from unstimulated controls) of three independent experiments ± S.D. of triplicates.

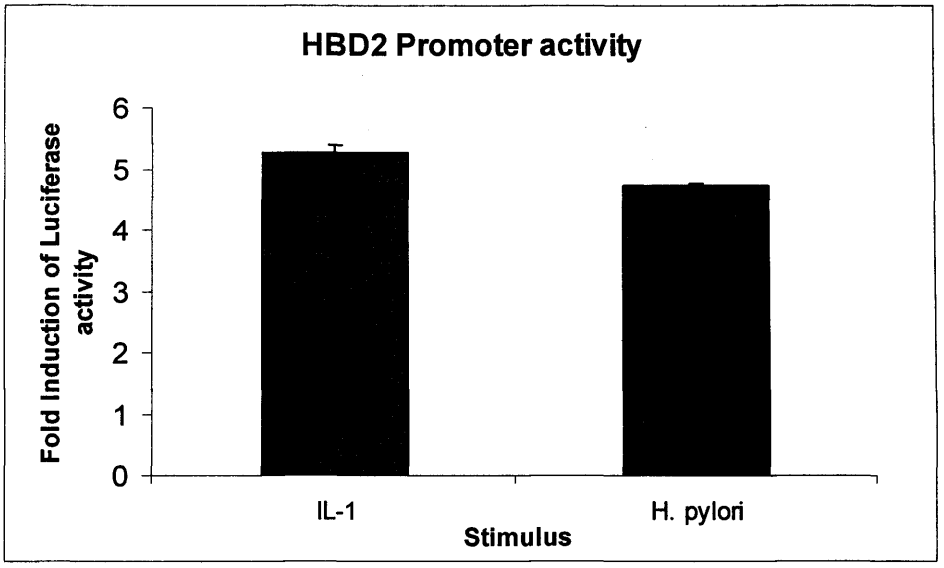


Figure 3.7 *H. pylori* induces hBD2 promoter activity in HEK 293 cells. HEK 293 cells were transiently co-transfected with pRL-TK-luciferase and pGL3-control vector and hBD2- luciferase reporter gene vector using Fugene transfection reagent. After 24h incubation, cells were stimulated with *H. pylori* (strain 60190 $1x10^8$cfu/ml) for 16h. Cells were harvested in passive lysis buffer and luciferase activity was measured as described above. Data represent the mean (fold induction from unstimulated controls) ± S.D. of triplicates.

Once hBD2 transfection conditions were established in HEK 293 cells, subsequent experiments were performed in AGS cells. Cells were co-transfected with innate immune promoter luciferase constructs (hBD2, hBD3 or IL-8), *Renilla* luciferase and pGL3-control (pcDNA 3.1) vector 24h prior to stimulation with IL-1β or wild type *H. pylori* for 8h (Figure 3.8). Promoter activities were analysed by luciferase reporter assay and fold increase in activity was compared to that of uninfected control cells. Importantly, in spite of lower transfection efficiency, AGS cells had a similar fold induction of promoter luciferase relative to *Renilla* luciferase activity to that seen in HEK 293 cells. IL-1β induced a 4 fold increase in hBD2 and IL-8 promoter activities (Figure 3.8) and a 2.5-3.5 fold induction was observed for *H. pylori*-mediated promoter activities for hBD2, hBD3 and IL-8 (Figure 3.8). The induction observed in promoter luciferase activity for IL-8, hBD2 and -3 matched results from the RT-PCR studies as IL-1β potently induced IL-8 and hBD2 expression whereas *H. pylori*-mediated expression was highest for hBD3.

Once the increase of β-defensin promoter activities by cytotoxic *H. pylori* was established, the role of bacterial virulence factors in promoter activation was investigated. The results of representative transfection experiments are shown in Figure 3.9. A significant ($p \leq 0.02$) increase in hBD2 promoter activity was observed during infection with *H. pylori* strains 60190 and 84-183. Infections in the presence of isogenic *cagA* and *vacA* mutant strains confirmed that these bacterial effectors play a minimal role in modulating epithelial innate immune responses, whereas the *cagE* mutant and strain Tx30a failed to elicit an hBD2 response (Figure 3.9a). Analysis of hBD3 promoter activity in response to a range of bacterial strains revealed a significant increase ($p < 0.05$) in the presence of *H. pylori* strains 60190 and 84-183 and the isogenic *vacA* and *cagA* mutant strains (Figure 3.9b). Interestingly, a modest increase in hBD3 promoter activity over control was observed for *cagE* mutant and strain Tx30a; however this increase did not reach statistical significance.

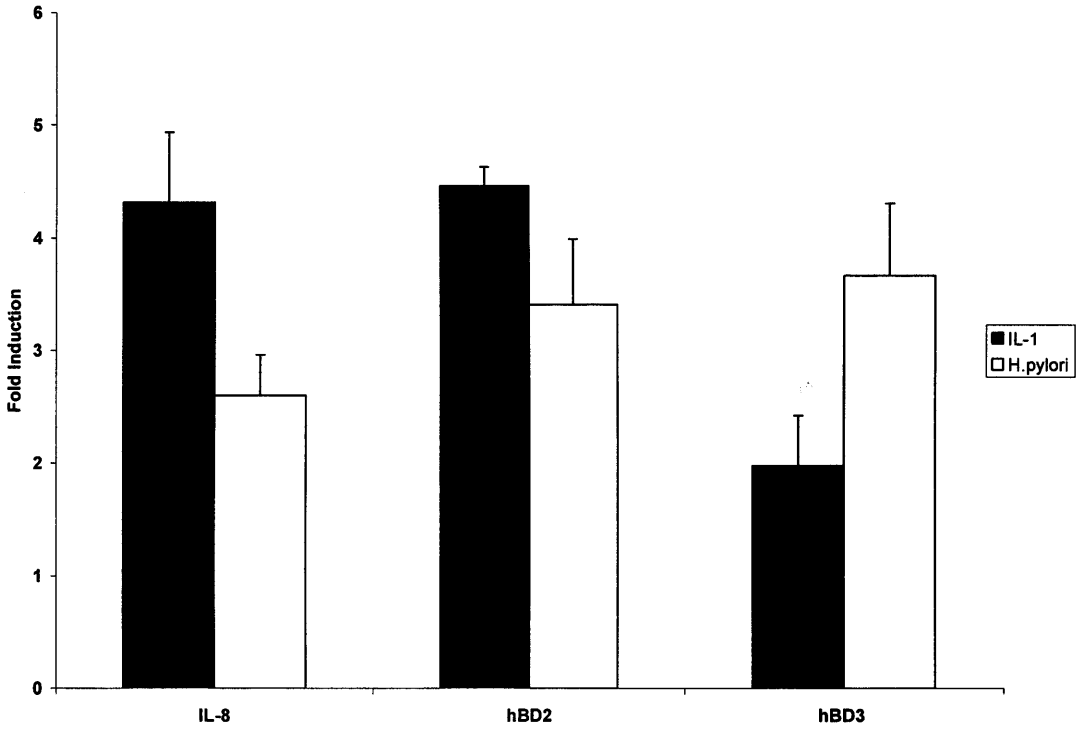
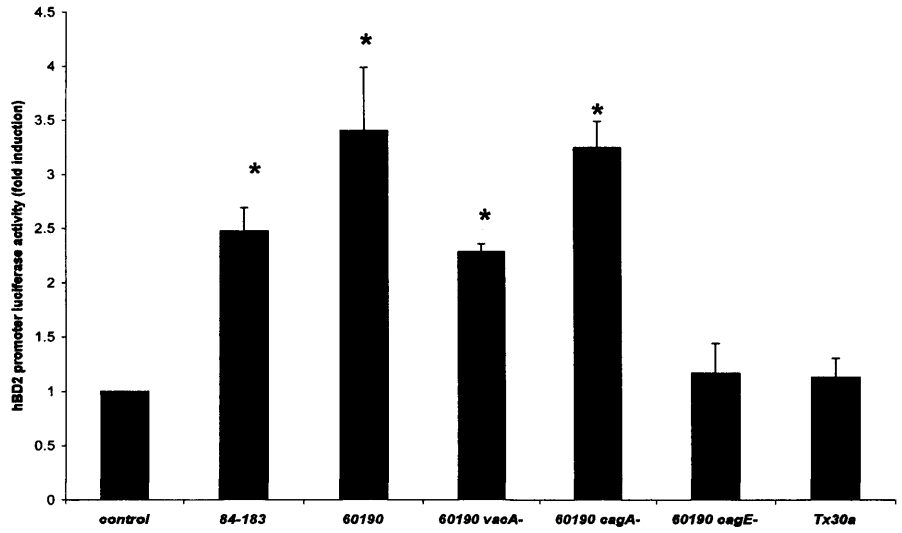


Figure 3.8 Innate immune promoter activities in AGS gastric epithelial cells.

AGS cells were transiently co-transfected with luciferase reporter gene plasmids (IL-8, hBD2 and hBD3), pRL-TK-luciferase and pGL3-control vector using Fugene transfection reagent. Cells were either infected with *H. pylori* (strain 60190 $1x10^8$cfu/ml) or stimulated with IL-1β (20ng/ml) for 16h after 24h. Cells were harvested in passive lysis buffer and luciferase activity was assayed. Data represent the mean fold induction from unstimulated controls ± S.D. of triplicates.

(a)



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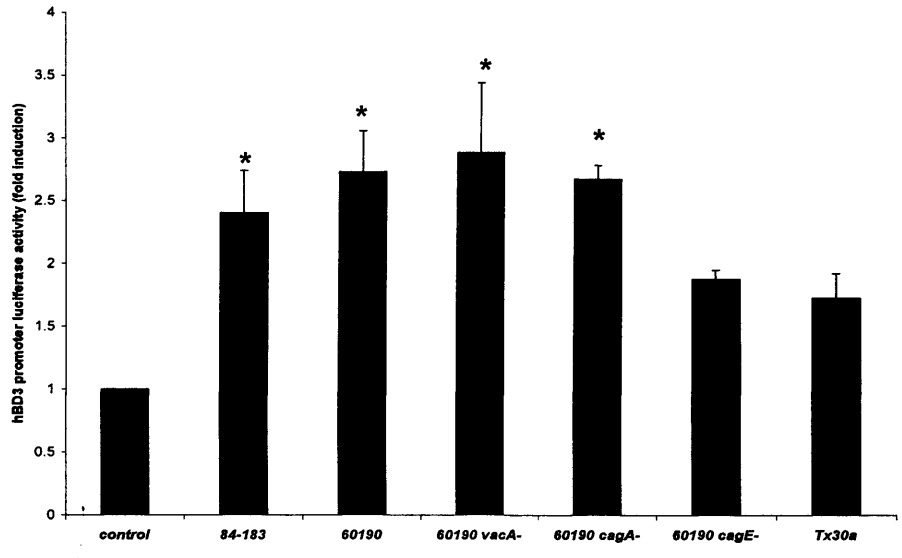
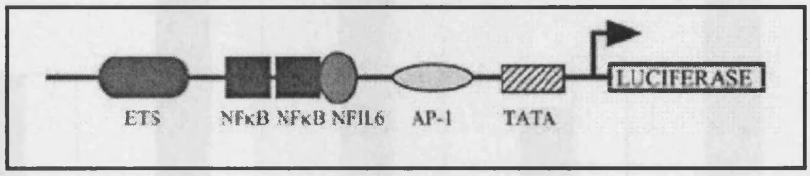


Figure 3.9: hBD2 and hBD3 promoter activity in response to infection.

AGS cells were transiently transfected with (a) hBD2 or (b) hBD3 promoter-luciferase constructs. Cells were exposed to wild type and isogenic mutant *H. pylori* strains for 8h. Data are expressed as n-fold increase in luciferase activity when compared to uninfected, control cells. Error bars indicate SEM of values representative of two to three experiments conducted in triplicates.

These findings suggest a minimal role for CagA and VacA as observed for the hBD2 promoter. Further, the increase in hBD3 promoter activity in the absence of a type IV secretion system (*CagE*, Tx30a) implicates the involvement of signalling events distinct from those involved in hBD2 gene regulation.

In order to identify specific elements responsible for the hBD2 promoter induction observed during *H. pylori* infection, a truncated construct containing the following binding sites (Lu *et al.*, 2004) was made available for this study (H. Kai, Kumamoto University, Japan).



Experiments were undertaken as before where transient transfection allowed for the introduction of the constructs into AGS cells. Cells were then exposed to two wild type strains (60190, 84-183), a *CagPAI-ve* strain (Tx30a) and IL-1β (Figure 3.10) for 8h before analysis of luciferase activities of the two constructs.

There was no significant difference observed between promoter activities regardless of the nature of the stimulation and the values were mirrored from approximately 3 fold induction with wild type to relatively no induction with Tx30a strain for both full length and truncated constructs (Figure 3.10). Since the truncated hBD2 (-247) promoter contains the main transcription factors involved in inflammatory responses including NF-κB, AP-1 and NF-IL6 (Lu *et al.*, 2004) it may be argued that these elements are sufficient for full activation of hBD2 promoter activity. However, since the contribution of any other factors present in the full length construct during *H. pylori* infection is not known, the full length promoter was utilised in subsequent experiments.

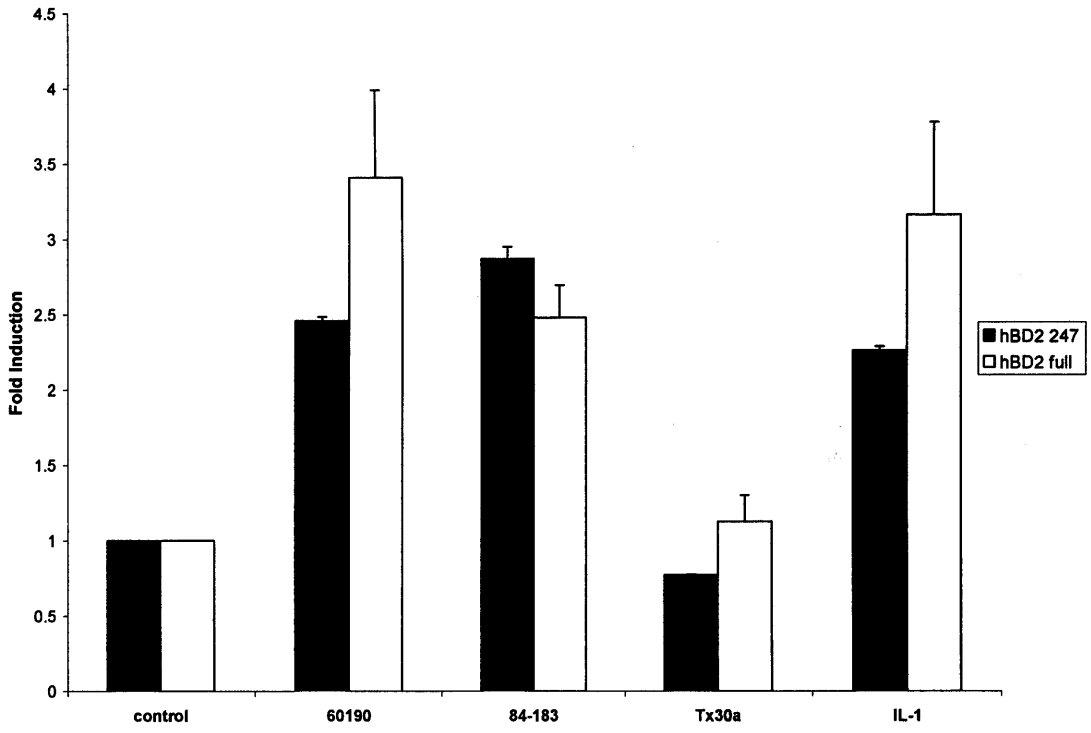


Figure 3.10 Full length and truncated (-247bp) hBD2 promoter constructs exhibit similar activity.

AGS cells were transiently transfected with full length (2kb) or truncated (247bp) promoter-luciferase constructs prior to exposure to wild type strains, Tx30a strain, or IL-1β for 8h.. No significant difference in activity was observed between the full length or truncated hBD2-luciferase promoter constructs. Data are expressed as n-fold increase in luciferase activity when compared to uninfected, control cells and error bars indicate SEM of values.

In conclusion, induction of hBD2 and hBD3 expression by wild type *H. pylori* strains both by mRNA and promoter analyses was found. Interestingly, a minimal role for the bacterial factors CagA and VacA in eliciting host innate epethial immune response was observed. We confirmed *Cag*PAI-dependent IL-8 expression and furthermore revealed COX-2 and IL-18 seem to be regulated by *Cag*PAI-independent mechanisms as mRNA levels remained unchanged during infection with an array of isogenic mutants.

Transient transfections utilising two separate cell-lines confirmed bacterial-mediated β-defensin induction as observed in earlier RT-PCR experiments. Both techniques suggested a critical role for the *Cag*PAI in hBD2 but not hBD3 gene regulation.

CHAPTER 4

Role of *H. pylori*-mediated NF-κB Activation in β-defensin Gene Regulation

4.0 Background

(a) Signalling pathways leading to NF-κB activation

Our understanding of host innate immune responses to infectious agents has leaped manifold in recent years. Research in the last decade has identified a series of pattern-recognition receptors (PRRs) that are intimately involved in recognising invariant structures or pathogen-associated molecular patterns (PAMPs) on micro-organisms (Akira and Takeda, 2004, Akira, 2006, Franchi *et al.,* 2006). The best characterised pattern recognition receptors include the Toll-like receptors (TLRs), a family comprising evolutionarily conserved, transmembrane proteins that recognise microbial components such as LPS, flagellin and peptidoglycan and have the ability to transduce signals leading to production of cytokines, chemokines and antimicrobial peptides (Biragyn *et al.*, 2002, Hertz *et al.*, 2003, Ozato *et al.*, 2002, Wang *et al.*, 2003b, Laube *et al.*, 2006, Pasare and Medzhitov, 2005). The extracellular domains of TLRs comprise leucine rich repeat (LRR) motifs and the cytoplasmic signalling domain shares significant homology with interleukin-1 receptor (IL- 1R) sequence, the homologous region is known as Toll/IL-1R (TIR) domain (Bowie and O'Neill, 2000). Initial interaction of ligand or PAMP and its specific PRR leads to (homo/hetero) dimerisation of TLRs, followed by recruitment of adaptor proteins (*via* TIR-TIR domain interactions). Adapter proteins include myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein inducing IFN-β (TRIF) or TRIF-related adaptor molecule (TRAM) (Akira *et al.*, 2006, Takeda and Akira, 2005). These adaptor molecules in turn may recruit IL-1R-associated kinases (IRAKs) and associate with tumour necrosis factor (TNF) receptor (TNFR)-associated factor (TRAF) 2, 3 or 6. Activation of several upstream kinases including IκB kinase (IKK), TANK-binding kinase (TBK)1 and TGFβ-activated kinase (TAK)1 culminates in phosphorylation of inhibitory IκB proteins. This event leads

to dissociation of these inhibitory proteins from the NF-κB complex, freeing the latter to translocate to the nucleus to function as a "transcription factor" (Ghosh and Karin, 2002, Karin, 2004).

(b) *H. pylori*-mediated NF-κB activation

It is well established that adherence of *H. pylori* to gastric epithelial cells activates multiple signal transduction events, leading to IL-8 gene transcription. Previous studies have shown that activation of transcription factor NF-κB plays a critical role in the regulation of host epithelial innate immune responses (Keates *et al.*, 1997, Sharma *et al.*, 1995, Glocker *et al.*, 1998, Munzenmaier *et al.*, 1997). Current understanding of *H. pylori*-mediated TLR signalling events in activated epithelial and/or monocytic cells is outlined in Figure 4.1.

The main TLR implicated in recognition of LPS of Gram-negative bacteria is TLR4. TLR 4 mRNA has been detected in human gastric epithelial cell-lines (Backhed *et al.*, 2003, Su *et al.*, 2003), however, the biological relevance of TLR4 receptor on human gastric cells remains unclear as cells are generally unresponsive to *H. pylori* LPS (Backhed *et al.*, 2003). Also, addition of a neutralising antibody to TLR4 does not block bacterial-mediated IL-8 production (Su *et al.*, 2003). The known low biological activity of *H. pylori* LPS (Moran *et al.*, 2005), combined with variation in expression of signalling components of the TLR4 pathway leaves the data at present inconclusive (Smith, Jr. *et al.*, 2003, Su *et al.*, 2003). In contrast to human studies, guinea pig gastric cells, do respond to *H. pylori* LPS, suggesting species variation (Kawahara *et al.*, 2001). Interestingly, unlike epithelial cells, effect of *H. pylori* LPS in NF-κB activation in human monoctyic cells is more convincing (Bhattacharyya *et al.*, 2002).

TLR2 plays an important role in the detection of several bacterial signature motifs including lipoteichoic acid (LTA), lipoproteins and peptidoglycan (PGN) (Akira *et al.*, 2006) with TLR5 responsible for the recognition of flagellin (Hayashi *et al.*, 2001). The

exact contribution of epithelial TLR2 and TLR5 in *H. pylori* detection remains ambiguous as human embryonic kidney (HEK) 293 cells show bacterial-mediated IL-8 expression in the absence of TLR2 (Backhed *et al.*, 2003, Schmausser *et al.*, 2004). Also *H. pylori* flagellin elicits minimal IL-8 activation despite expression of functional TLR5 in adult gastric epithelium (Gewirtz *et al.*, 2004). A recent investigation detailing *H. pylori* and *Campylobacter jejuni* flagellin suggest specific mutations in the flagellin molecule has obliterated TLR5 detection, whilst compensatory changes have allowed preservation of flagellar motility (Andersen-Nissen *et al.*, 2005).

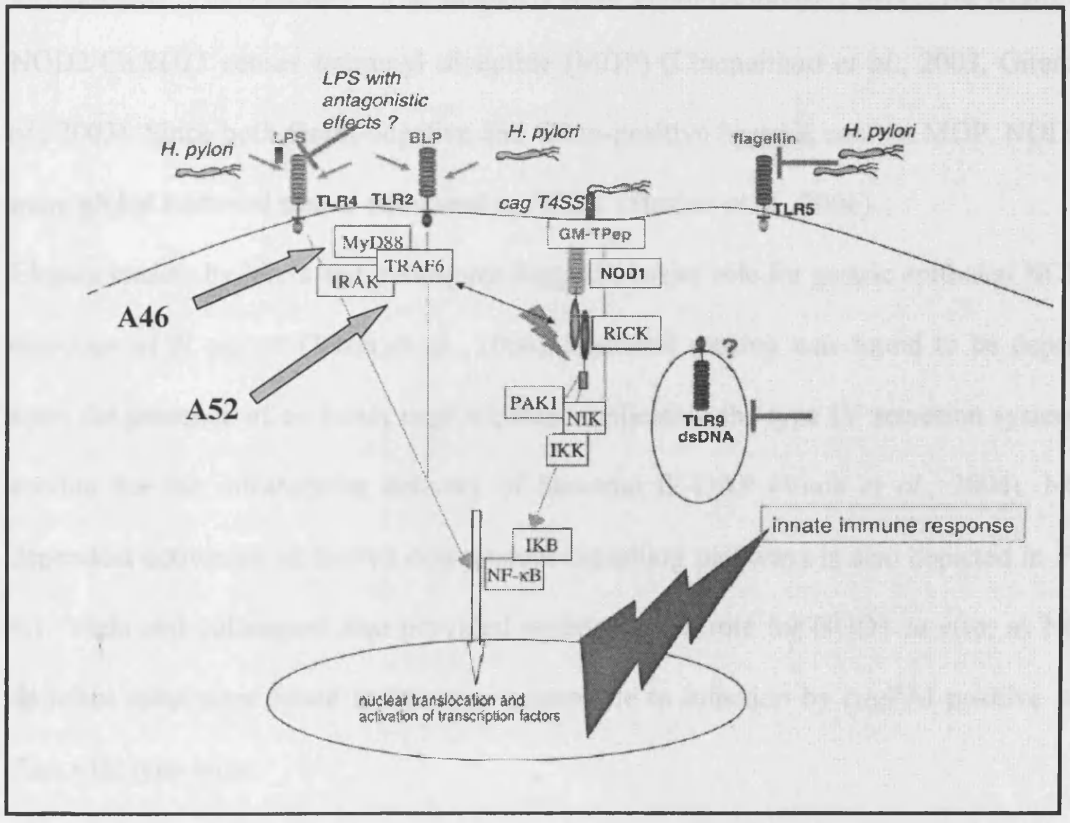


Figure 4.1 Model of *H. pylori cag*PAI-dependent and –independent activation of host epithelial innate immune responses (adapted from Lee and Josenhans, 2005). Downstream signalling blockage by A46 and A52 viral proteins is indicated (Bowie *et al*, 2000).

This clearly provides an immune evasion mechanism for the bacteria without compromising motility, a process essential for successful colonisation and pathogenesis.

A second family of PRRs, cytoplasmic nucleotide-binding oligomerisation domain (NOD) proteins, have also been implicated in epithelial host innate immune responses to infection. NOD1 (encoded by the caspase-recruitment domain 4 gene, *CARD4*) and NOD2 (encoded by *CARD15*) recognise peptidoglycan (PGN), a component of bacterial cell walls and like the TLRs, rely on recognition of pathogens by a leucine rich repeat (LRR) domain (Inohara and Nunez, 2003, Inohara *et al.*, 2005, Franchi *et al.,* 2006). Specifically, NOD1/*CARD4* distinguishes its ligand, γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) and NOD2/*CARD15* senses muramyl dipeptide (MDP) (Chamaillard *et al.*, 2003, Girardin *et al.*, 2003). Since both Gram-negative and Gram-positive bacteria contain MDP, NOD2 is a more global bacterial sensor compared to NOD1 (Strober *et al.,* 2006).

Elegant studies by Viala and colleagues suggest a major role for gastric epithelial NOD1 in detection of *H. pylori* (Viala *et al.*, 2004). Bacterial sensing was found to be dependent upon the presence of an intact *cag*PAI; thus implicating the type IV secretion system as a conduit for the intracellular delivery of bacterial iE-DAP (Viala *et al.*, 2004). NOD1-dependent activation of known downstream signalling pathways is also depicted in Figure 4.1. Viala and colleagues also provided evidence for a role for NOD1 *in vivo,* as NOD1-deficient mice were found to be more susceptible to infection by *cag*PAI positive strains than wild type mice.

(c) *H. pylori*-mediated β-defensin expression

The role of hBDs in *H. pylori* infection both *in vitro* and *in vivo* has been recently evaluated (Bajaj-Elliott *et al.*, 2002, George *et al.*, 2003, Hamanaka *et al.*, 2001, Lee and Josenhans, 2005, O'Neil *et al.*, 2000, Wada *et al.*, 2001). Although, hBD1 expression is constitutive, it can be further modulated *in vitro* and *in vivo* in response to *H. pylori* (Bajaj-Elliott *et al.*,

2002). In contrast, hBD2 expression is induced by *H. pylori* infection (Bajaj-Elliott *et al.*, 2002, O'Neil *et al.*, 2001). Furthermore hBD2 induction in MKN45 gastric cells was found to be up-regulated via participation of the *Cag*PAI in a NF-κB-dependent manner (Wada *et al.*, 1999, Wada *et al.*, 2001). hBD2 regulation has been studied in detail revealing four NF-κB binding sites involved in its transcriptional regulation. Several studies suggest that the proximal NF-κB binding site is critical for hBD2 gene expression in intestinal and gastric epithelia (Figure 4.2; Ogushi *et al.*, 2004, Tsutsumi-Ishii and Nagaoka, 2003, Vora *et al.*, 2004, Wada *et al.*, 2001, Wehkamp *et al.*, 2004).

Figure 4.2 NF-κB and AP-1 contribute to hBD2 transcriptional regulation.

Previous work has also shown that although *H. pylori* can activate p65-p65, p50-p50 and p65-p50 homo- and hetero-dimer formation, it is the p65-p65 homodimer that binds to hBD2 promoter region (Wada *et al.*, 2001). Although a role for NF-κB in bacterial mediated hBD2 expression is established, upstream signalling events leading to NF-κB activation and hBD2 induction during *H. pylori* infection have not been studied.

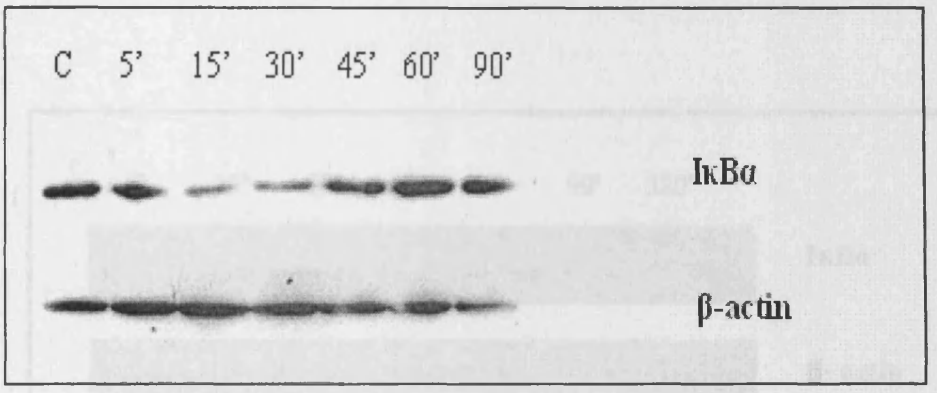
Further, to date, no studies have investigated the regulation of hBD3 expression during *H. pylori* infection. Up-regulation of hBD3 gene expression by wild type bacteria found in chapter three has now been reported (George *et al.*, 2003).

4.1 A major role for IκBα but not IκBβ in *H. pylori*-mediated NF-κB activation

Phosphorylation and subsequent degradation of IκB proteins is a well-established upstream event prior to NF-κB nuclear translocation. Although several studies have previously shown IκBα degradation in response to *H. pylori*, confirmation of this event was sought in this co-culture system, mainly to validate the infective nature of the wild-type bacterial strains being utilised in this thesis. Secondly, degradation of IκBα would be indicative of integrity of NF-κB associated pathways in AGS cells- the choice of gastric cell-line employed. Detectable levels of IκBα were present in control unstimulated AGS cells (Figure 4.3-4.5). Exposure to two cytotoxic wild type *H. pylori* (60190 and 84-183) strains resulted in rapid (15-30min) IκBα degradation with protein levels returning to that of control by 60min (Figure 4.3). These findings are comparable to those of Nozawa *et al*, 2002, where *H. pylori* strain 43504 was investigated. Although several studies have shown *H. pylori*-mediated NF-κB activation (Bhattacharyya *et al.*, 2002, Glocker *et al.*, 1998, Keates *et al.*, 1997, Nozawa *et al.*, 2002, Wada *et al.*, 2001), this study is the first detailing kinetics of IκBα degradation. Stimulation of AGS cells by IL-1β (positive control) also led to degradation of IκBα, interestingly, the kinetics of IκBα degradation by IL-1β paralleled those observed in *H. pylori* treated cells (Figure 4.3 compared with Figure 4.4).

Most studies to date have concentrated on the role of IκBα in *H. pylori*-mediated NF-κB activation, however, in cells nearly half of NF-κB resides complexed to another major inhibitory protein isoform, IκBβ (Whiteside *et al.*, 1997). The potential role of IκBβ in *H. pylori*-mediated NF-κB activation was assessed. For this purpose, IκBβ expression during infection was followed. Level of IκBβ in unstimulated, control AGS cells was found to be much lower than that observed for IκBα (Figure 4.3 *versus* Figure 4.5). No IκBβ degradation was observed either during cytokine or bacterial stimulation, this effect was followed up to 4h post-activation (Figure 4.5). Interestingly, instead of degradation increase

(a)



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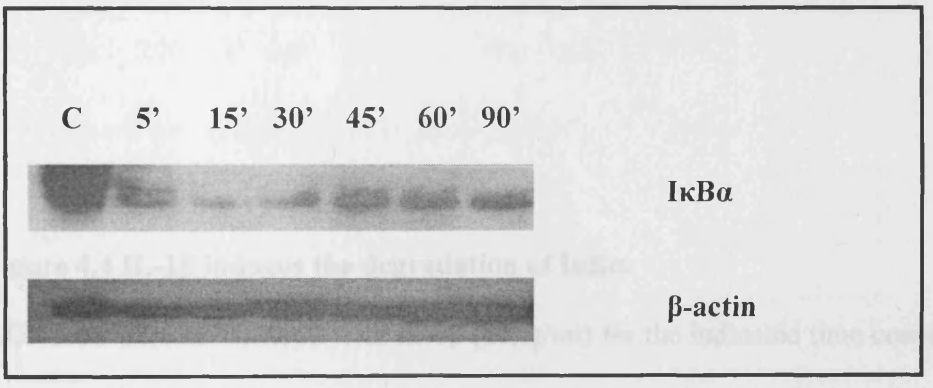


Figure 4.3 Rapid degradation of inhibitory protein IκBα in *H. pylori* infection.

AGS cell monolayers were infected with live wild type cytotoxic strains (a) 60190 and (b) 84183 ($1x10^8$ cfu/ml). Time course of NF-κB activation was evaluated by subjecting cell lysates to SDS-PAGE and Western Blotting. Equal protein loading was confirmed by reprobing the blot with a β-actin antibody. Experiments were conducted three times and a chosen representative blot is shown.

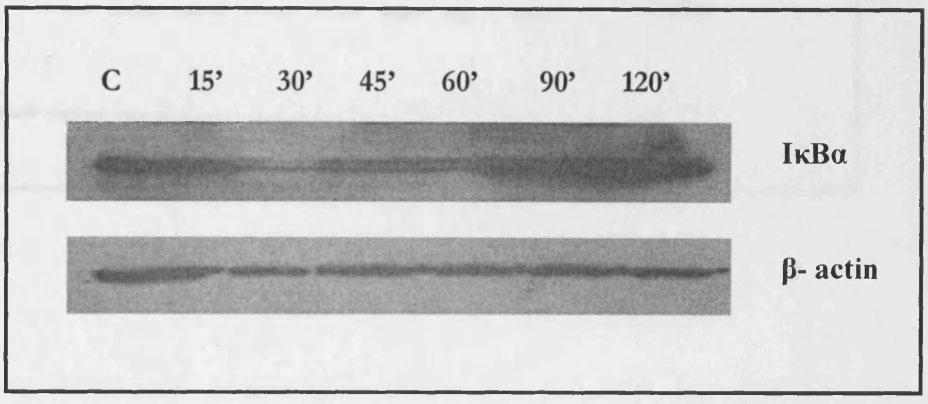
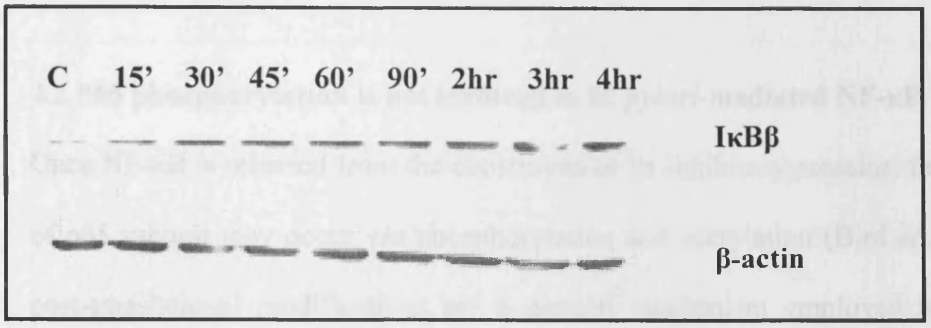


Figure 4.4 IL-1β induces the degradation of IκBα.

AGS cells were co-cultured with IL-1β (20ng/ml) for the indicated time course. Cell lysates were subjected to SDS-PAGE, Western Blotting and activation of NF-κB was followed by degradation of IκBα. Equal protein loading was confirmed by reprobing the blot with a β-actin antibody. Experiments were conducted three times and a chosen representative blot is shown.

(a)



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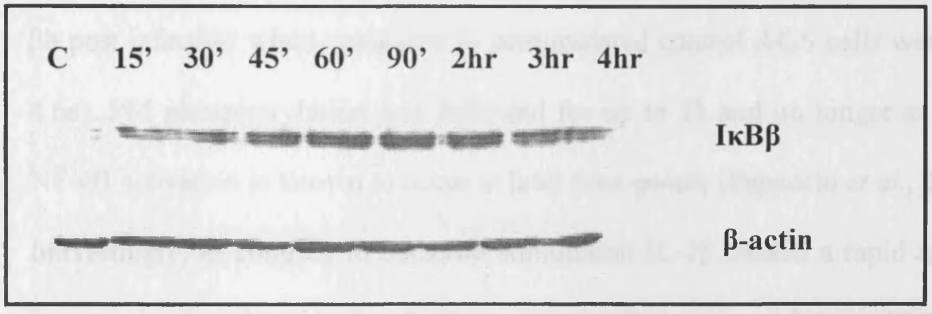


Figure 4.5 Inhibitory protein, IκBβ is not degraded during *H. pylori* infection. Cell lysates from (a) *H. pylori* (60190; $1x10^8$ cfu/ml) infected AGS cells were subjected to SDS-PAGE and Western Blotting. No obvious degradation of IκBβ was observed up to 4h post-infection or (b) IL-1β (20ng/ml) stimulation. Experiments were conducted three times and a chosen representative blot is shown.

in the amount of IκBβ at 60-90min with IL-1β, and 3-4h post-bacterial infection was observed.

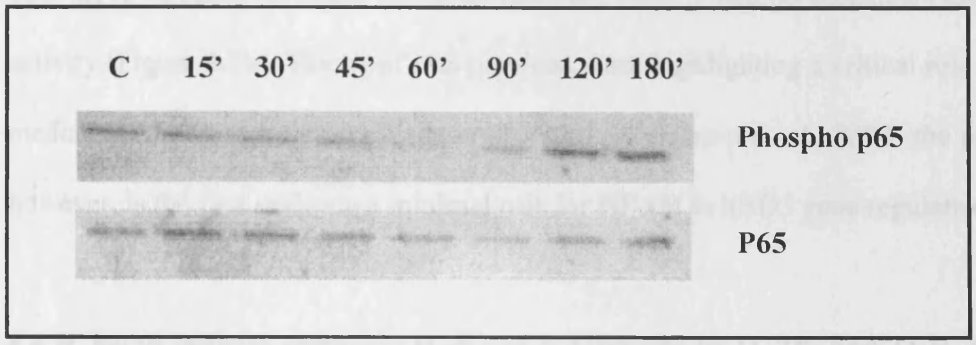
4.2 P65 phosphorylation is not involved in *H. pylori*-mediated NF-κB activation

Once NF-κB is released from the constraints of its inhibitory proteins, further modification of p65 subunit may occur *via* phosphorylation and acetylation (Bird *et al.*, 1997). Protein post-translational modifications are a general mechanism employed to regulate protein function. To date, no studies have reported epithelial p65 phosphorylation status during *H. pylori* infection. In the present study, p65 phosphorylation status was investigated in our established *in vitro* model system. A modest increase in the levels of phosphorylated p65 2-3h post infection when compared to unstimulated control AGS cells was observed (Figure 4.6a). P65 phosphorylation was followed for up to 3h and no longer as a second wave of NF-κB activation is known to occur at later time-points (Papaccio *et al.*, 2005).

Interestingly, in contrast to bacterial stimulation IL-1β caused a rapid and potent transient increase in phosphorylated p65 which was noted as early as 30min (Figure 4.6b). Maximal levels were attained at 60min after stimulation. These experiments suggest differential regulation of NF-κB activation in response to cytokine and bacterial stimuli.

4.3 NF-κB is involved in the regulation of hBD2 and IL-8 but not hBD3 gene expression

To evaluate any potential contribution of NF-κB in IL-8 and β-defensin gene regulation, infections in the presence of MG132, a specific proteasome inhibitor were conducted. AGS cells pre-treated with MG132 were exposed to *H. pylori* for 8h and samples analysed by RT-PCR and promoter luciferase activity. There was a striking inhibition of hBD2 and IL-8 expression in the presence of MG132 compared to cells infected with bacteria alone (Figure 4.7a), this is likely to be related to the inhibitors ability to prevent proteasomal degradation



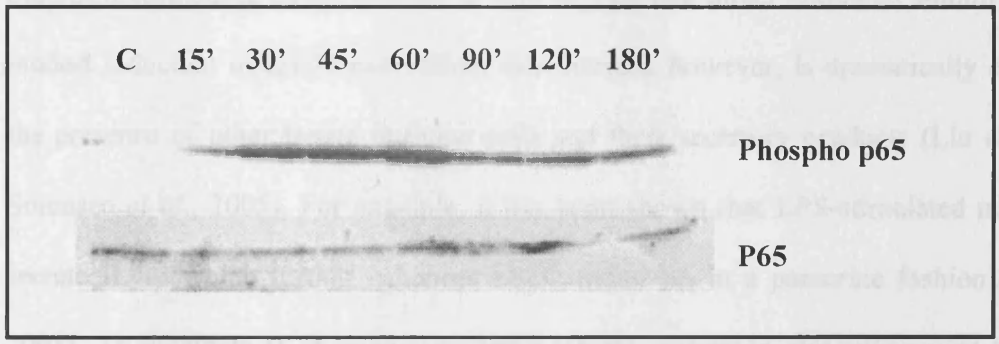


Figure 4.6 Evaluation of p65 phosphorylation during *H. pylori*-mediated NF-κB activation. Infection with (a) *H. pylori* (strain HP 60190, $1x10^8$cfu/ml) or stimulation with (b) IL-1β (20ng/ml) was carried out for up to 3h. Whole cell lysates were subjected to Western Blotting for phospho-specific p65. A non-phospho p65 antibody was used to demonstrate equal levels of p65 protein. Experiments were conducted at least three times and a chosen representative blot is shown.

of IκB proteins. A dramatically significant reduction in hBD2 (>80% inhibition) promoter activity ($p<0.05$; Figure 4.7b) and more than 60% inhibition for IL-8 was also observed. In contrast to its effect on hBD2 promoter function, MG132 had no significant effect on hBD3 activity (Figure 4.7b). This confirms previous work highlighting a critical role of NF-κB in mediating hBD2 expression (Wada *et al.*, 2001, Wehkamp *et al.*, 2004) the present study, however, is the first defining a minimal role for NF-κB in hBD3 gene regulation.

4.4 *H. pylori* induces epithelial IL-8 and β-defensins by IL-1β- and TLR- independent mechanisms

Studies investigating innate defence in skin suggest that direct microbial stimuli results in a modest induction in hBD2 expression; this increase however, is dramatically enhanced in the presence of other innate immune cells and their secretory products (Liu *et al.*, 2003, Sorensen *et al.*, 2005). For example, it has been shown that LPS-stimulated macrophages secrete IL-1β which further enhances hBD2 induction in a paracrine fashion (Liu *et al.*, 2003). To elucidate if *H. pylori*-mediated NF-κB activation and subsequent hBD2 gene expression was a result of direct microbial assault or an indirect, autocrine effect of IL-1β release during infection, several approaches were followed. Firstly, any potential induction of IL-1β mRNA in our co-culture experiments was investigated, no expression of IL-1β during the 24h period of infection was observed (data not shown). Secondly, the effect of exogenous IL-1 receptor antagonist (IL-1RA) was evaluated by conducting cytokine and bacterial stimulation of AGS cells in the presence and absence of IL-1RA (Figure 4.8). In the presence of IL-1β or *H. pylori* an increase in hBD2 was noted compared to unstimulated control cells, with greater induction of hBD2 in response to IL-1β (Figure 4.8; lane B). Inclusion of IL-1RA caused a dramatic inhibition of hBD2 gene expression in IL-1β-stimulated cells (Figure 4.8; lane B compared to lane C), whereas *H. pylori*-mediated hBD2

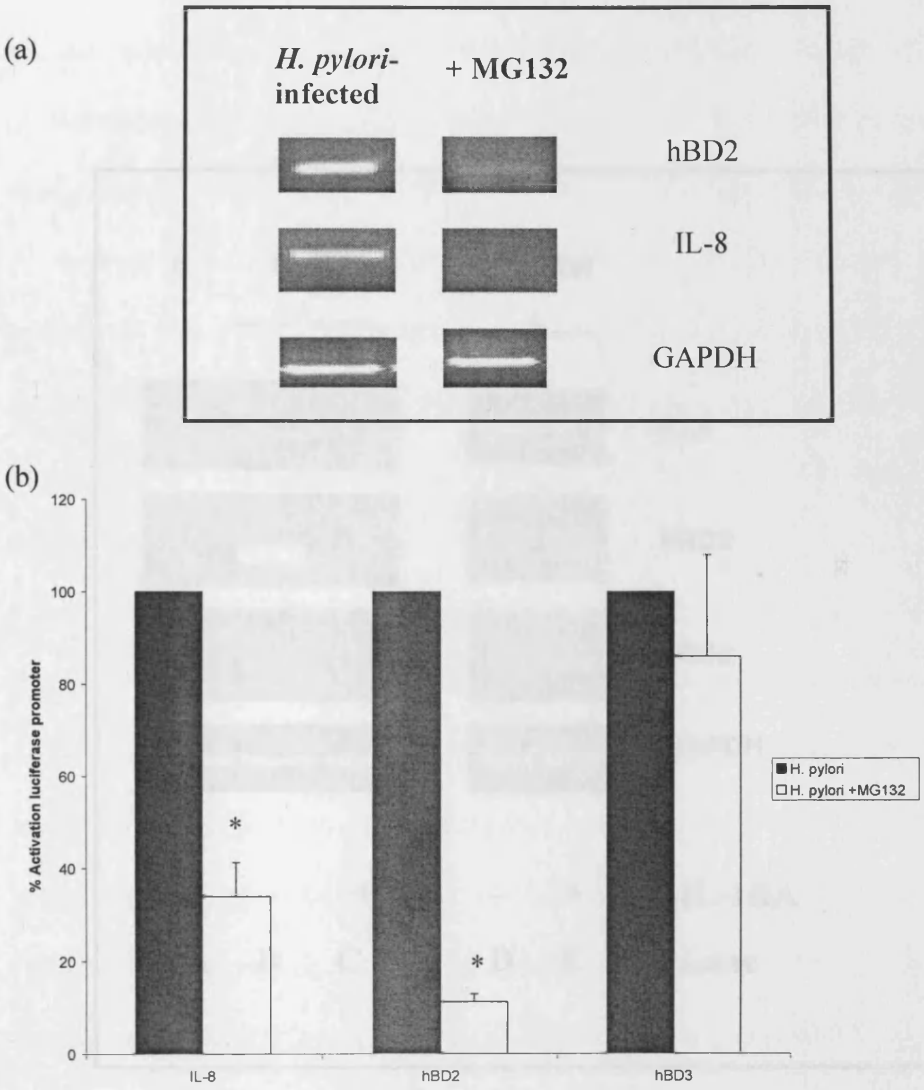


Figure 4.7 A potential role of NF-κB activation in *H. pylori*-mediated IL-8 and hBD2 but not hBD3 gene regulation.

(a) Cells were pre-treated with 25μM MG132, a specific proteasome inhibitor, prior to exposure to wild type *H. pylori* strain 60190 for 8h and RT-PCR analyses were conducted. A representative of three independent experiments is shown. (b) Transient transfection experiments were undertaken introducing promoter-luciferase constructs into AGS cells 24h prior to inhibitor pre-treatment and infection with *H. pylori*. Significant (* $p<0.05$) reduction of IL-8 and hBD2 promoter activities was observed. Experiments were conducted in triplicate and repeated three times.

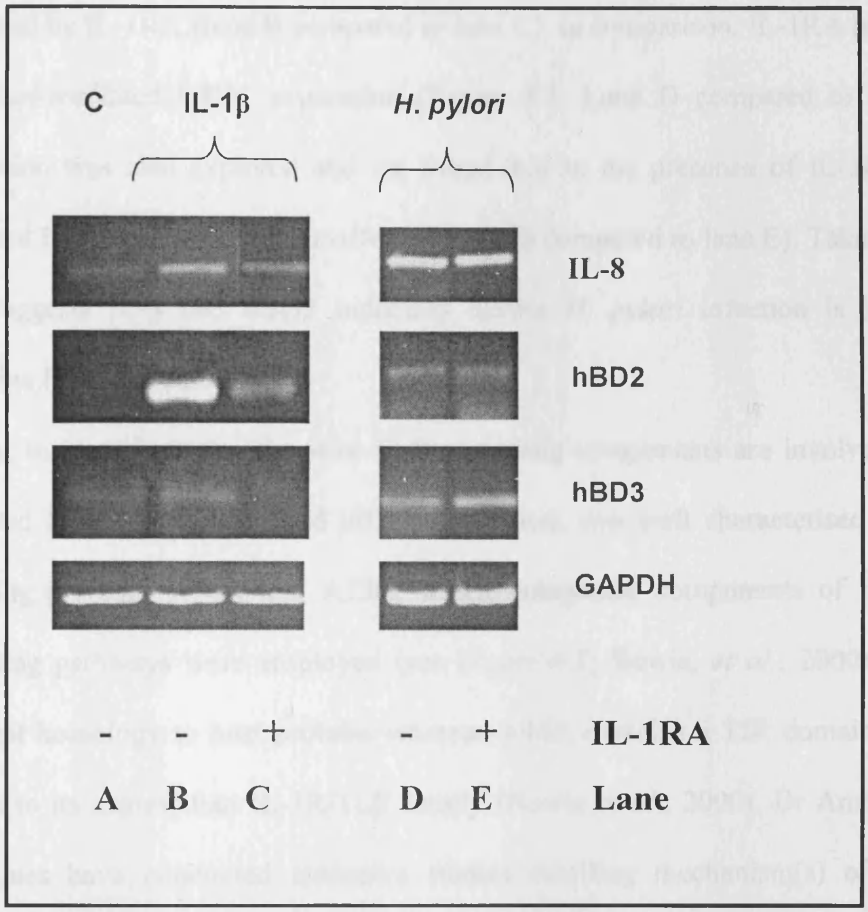


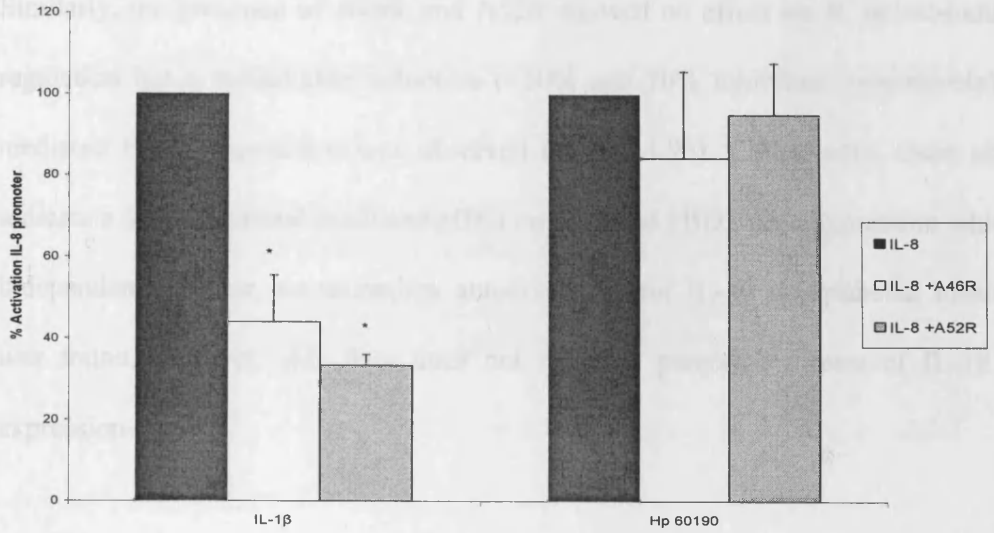
Figure 4.8: *H. pylori*–mediated innate immune gene expression is independent of IL-1β.

AGS cells were stimulated with IL-1β (20 ng/ml) or *H. pylori* ($1x10^8$ cfu/ml) for 8h both in the presence or absence of IL-1RA (200 ng/ml). Innate immune gene expression was assessed by RT-PCR. A representative analysis of at least 3 independent experiments is shown.

expression remained unchanged in the presence of the receptor antagonist (Figure 4.8; lane D compared to lane E). IL-1β caused a modest increase in hBD3 expression which was abolished by IL-1RA (lane B compared to lane C); in comparison, IL-1RA had no effect on *H. pylori*-mediated hBD3 expression (Figure 4.8; Lane D compared to Lane E). IL-8 expression was also explored and we found that in the presence of IL-1RA, *H. pylori*-mediated IL-8 expression was unaffected (lane D compared to lane E). Taken together, this data suggests IL-8 and hBD2 induction during *H. pylori* infection is independent of autocrine IL-1β production.

Finally, to identify if IL-1β and/or TLR-signalling components are involved in *H. pylori*-mediated NF-κB activation and hBD2 expression, two well characterised *Vaccinia* virus encoding proteins, A46R and A52R, which antagonise components of IL-1β and TLR signalling pathways were employed (see Figure 4.1; Bowie, *et al.*, 2000). A52R has no apparent homology to host proteins whereas A46R contains a TIR domain, thus distantly related to its mammalian IL-1R/TLR family (Bowie *et al.*, 2000). Dr Andrew Bowie and colleagues have conducted extensive studies detailing mechanism(s) of viral immune evasion and have revealed that A46R and A52R block IL1R/TLR-mediated NF-κB activation. Specifically, A52R disrupts host signalling by associating with TRAF6 and IRAK2 and A46R targets TIR-domain containing adaptor molecules such as MyD88 and MAL (Figure 4.1; Harte *et al.*, 2003, Stack *et al.*, 2005). Either way both viral proteins prevent downstream signalling and NF-κB activation. Plasmids encoding A46R and A52R were co-transfected with IL-8 and hBD2 promoter-constructs prior to stimulation with *H. pylori* or IL-1β (Figure 4.9). If there was paracrine IL-1β secretion or the TLRs were involved in the induction of hBD2, one would expect the antagonists to prevent up-regulation of hBD2 gene expression. A significant reduction of IL-8-luciferase activity in the presence of A46R and A52R was observed ($p<0.05$) in IL-1β-stimulated cells but there

(a)



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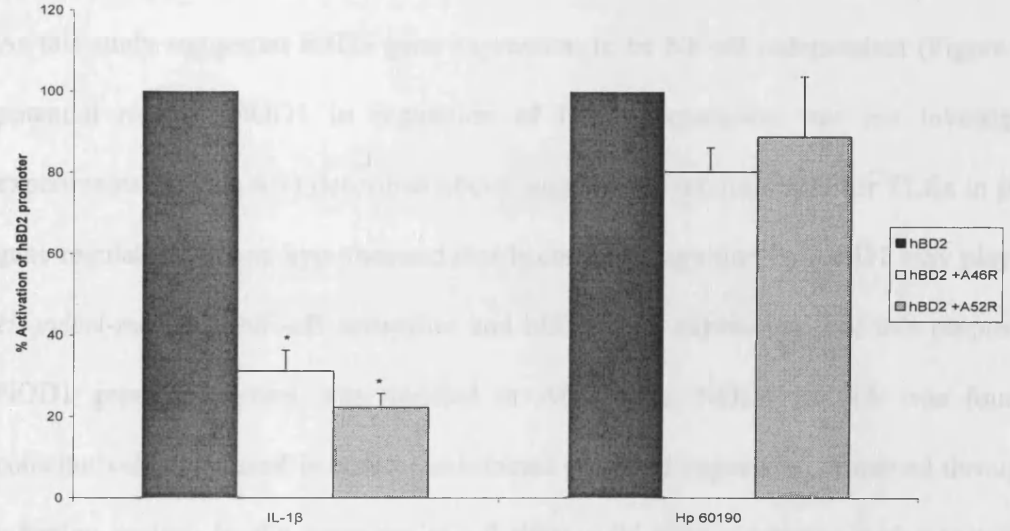


Figure 4.9 *H. pylori*- mediated innate immune gene expression is independent of IL-1β or TLR- signaling pathways.

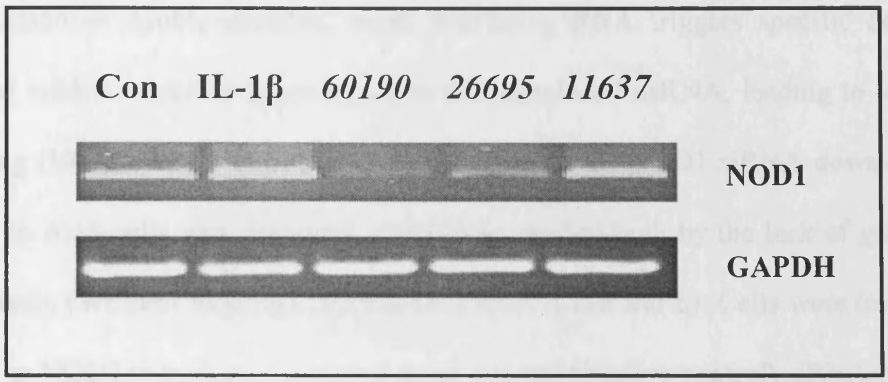
AGS cells were transiently transfected with A46R and A52R plasmids along with (a) IL-8 or (b) hBD2 promoter construct for 24h prior to bacterial or cytokine stimulation. Values are expressed as mean ±SEM from a representative of three experiments conducted in triplicates. Inhibition of IL-1β stimulation in the presence of both A46R and A52R was statistically significant * $p < 0.05$.

was no significant effect on *H. pylori*-mediated IL-8 promoter function (Figure 4.9a). Similarly, the presence of A46R and A52R showed no effect on *H. pylori*-induced hBD2 regulation but a remarkable reduction (<60% and 70% inhibition respectively) on IL-1β mediated hBD2 expression was observed (Figure 4.9b). Collectively, these observations indicate a direct bacterial-mediated effect on IL-8 and hBD2 gene expression which is TLR-independent. Further, no secondary autocrine role for IL-1β on epithelial innate response was found, however, this data does not rule out paracrine effects of IL-1β on hBD2 expression *in vivo*.

4.5 NOD1: a critical player in hBD2 but not hBD3 gene regulation

As this study suggested hBD3 gene expression to be NF-κB independent (Figure 4.7), any potential role of NOD1 in regulation of hBD3 expression was not investigated. As experiments (Figure 4.9) described above suggested a minimal role for TLRs in β-defensin gene regulation, it was hypothesised that bacterial recognition by NOD1 may play a role in *H. pylori*-mediated NF-κB activation and hBD2 gene expression. For this purpose, firstly, NOD1 gene expression was verified in AGS cells. NOD1 mRNA was found to be constitutively expressed in control uninfected cells and expression remained throughout the infection period, in the presence of all three wild type cytotoxic strains tested (60190, 26695, 11637; Figure 4.10a). However, varying levels of NOD1 mRNA expression were noted for the different wild type strains; 60190, 26695 and 11637 (Figure 4.10a). Variation in NOD1-expression during infection (compared to control) may represent inter-experimental variation and needs to be further verified by more quantitative techniques e.g. quantitative PCR and Western blotting. Importantly, infection in the presence of *cagA, vacA* and *cagE* isogenic mutants had minimal effect on NOD1 expression, suggesting these virulence determinants do not modulate NOD1 expression as an immune evasion strategy.

(a)



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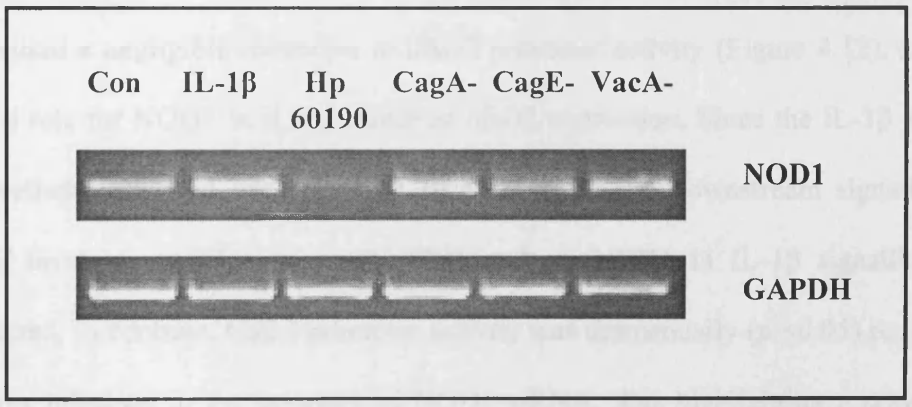


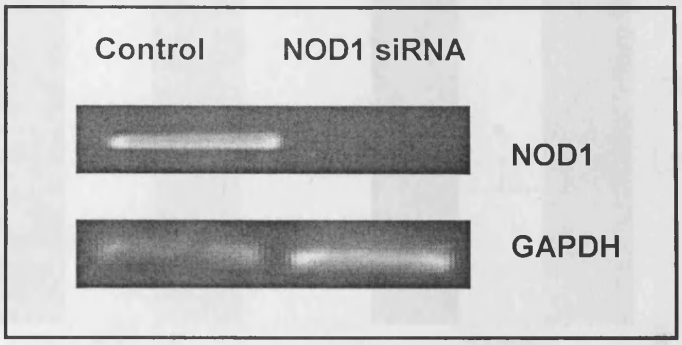
Figure 4.10 NOD1 mRNA is expressed in AGS cells. Cells were either left unstimulated or exposed to three wild type cytotoxic strains of *H. pylori* ($1x10^8$ cfu/ml) for 8h prior to assessment by RT-PCR (a). NOD1 expression was detected in control and cells infected with isogenic mutants (b).

NOD2 mRNA was not detected in control or infected AGS cells (data not shown). Next, experiments utilising a NOD1 siRNA encoding plasmid were conducted.

Introduction of double-stranded, small interfering RNA triggers specific degradation of targeted mRNA which is homologous to the introduced dsRNA, leading to effective gene silencing (Novina and Sharp, 2004). In the presence of NOD1 siRNA down-regulation of NOD1 in AGS cells was observed, which was verified both by the lack of gene (RT-PCR) and protein (Western blotting) expression (Figure 4.11a and b). Cells were transfected with NOD1 or NOD2 (which was included as an internal negative control) siRNA plasmids prior to transfection of hBD2 or hBD3 promoter constructs. After further incubation, cells were exposed to appropriate stimuli. The presence of NOD1 or NOD2 siRNA in IL-1β stimulated cells caused a negligible reduction in hBD2 promoter activity (Figure 4.12), suggesting a minimal role for NOD1 in IL-1β mediated hBD2 expression. Since the IL-1β pathway has been well-characterised with the IL-1 receptor mediating downstream signalling events, lack of involvement of a microbial PRR such as NOD1 in IL-1β signalling was not unexpected. In contrast, hBD2 promoter activity was dramatically ($p < 0.05$) reduced during *H. pylori* infection in the presence of NOD1 siRNA, thus highlighting a critical role for NOD1 in *H. pylori*-induced hBD2 gene expression. When similar experiments were conducted in the presence of the hBD3 promoter construct no effect of NOD1 or NOD2 siRNA on IL-1β- or bacterial-mediated hBD3 expression was found (Figure 4.12b).

In addition, experiments were also executed utilising commercially available siRNA (Ambion Europe Ltd, Huntingdon, UK) targeting human NOD1 to complement results obtained in the presence of NOD1 siRNA plasmid (Figure 4.13). In the presence of NOD1 siRNA (referred to as ii in Figure 4.13), *H. pylori*-mediated hBD2 expression was significantly reduced (Figure 4.13), as observed with the siNOD1-encoding plasmid (Figure 4.12). No significant difference was detected in hBD3 promoter activity (Figure 4.13),

(a)



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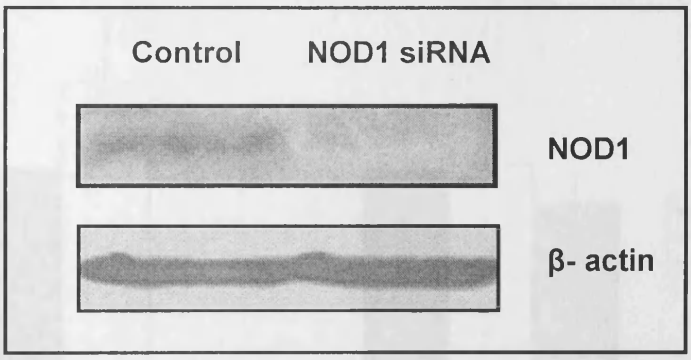
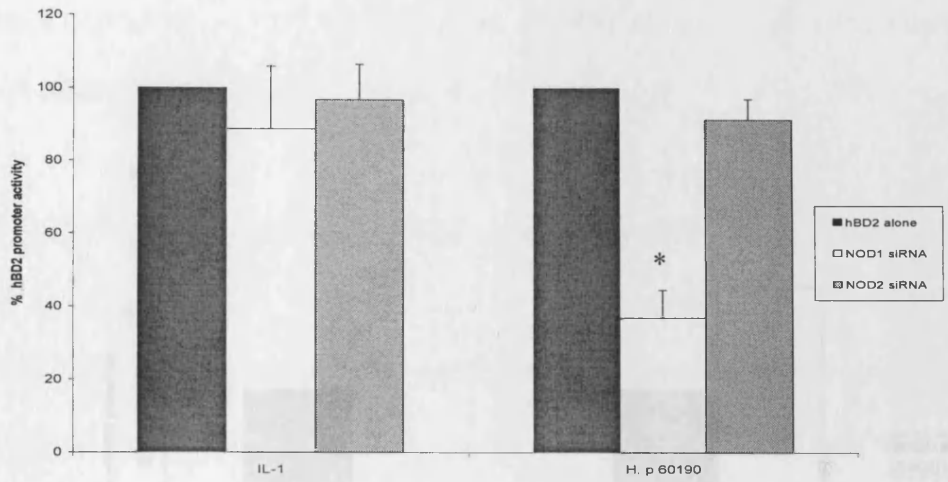


Figure 4.11 Small interfering NOD1 down-regulates NOD1 gene and protein expression. AGS cells were transfected with NOD1 siRNA encoding plasmid for 24h prior to evaluation of (a) NOD1 mRNA and (b) protein.

(a)



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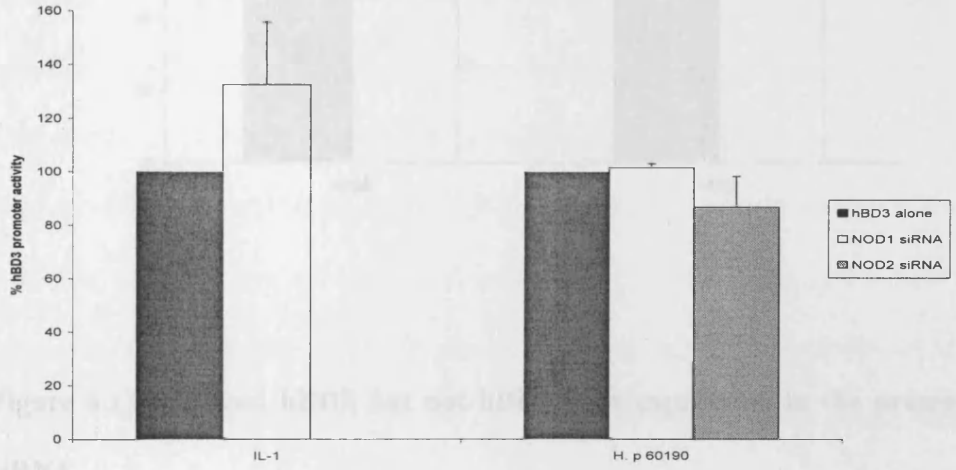


Figure 4.12 NOD1 plays major role in hBD2 but not hBD3 expression.

AGS cells were transiently transfected with a plasmid encoding NOD1 or NOD2 siRNA 24h prior to introduction of β-defensin promoter constructs and 24h later, cells were stimulated with IL-1β or *H. pylori* for 8h. Luciferase activities for (a) hBD2 and (b) hBD3 were evaluated and error bars indicate SEM of values obtained from three independent experiments conducted in triplicates. Statistical significance was determined by t- test, * $p < 0.05$.

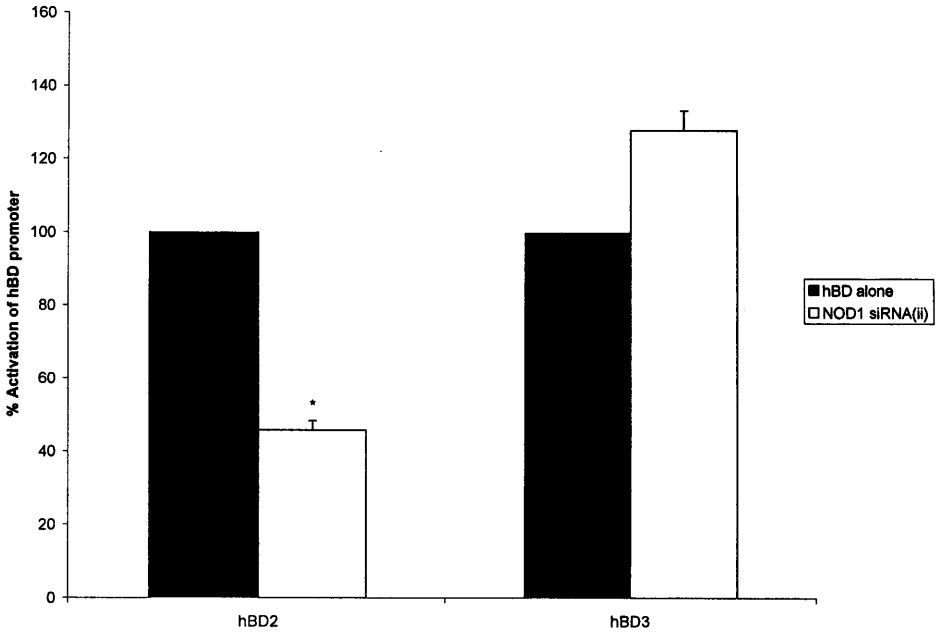


Figure 4.13 Reduced hBD2 but not hBD3 gene expression in the presence of NOD1 siRNA.

AGS cells reverse transfected with siRNA for NOD1 (ii) 48h prior to the introduction of β-defensin promoter constructs and 24h later, cells were infected with *H. pylori* for 8h. Luciferase activities for hBD2 and hBD3 were evaluated and error bars indicate SEM of values obtained from three independent experiments conducted in triplicates. Statistical significance was determined by t- test, * $p < 0.05$.

suggesting a major role for NOD1 in hBD2 but not hBD3 gene regulation. The present study is the first identifying NOD1 as a major signal transduction player involved in differential β-defensin gene expression.

4.6 NOD1 knock-out mice exhibited impaired murine β-defensin 4 expression during *H. pylori* infection

This series of experiments were performed by Professor G. Núñez and colleagues at the University of Michigan as part of a collaborative study. I am grateful to Professor Núñez for allowing me to include Figure 4.14 in this thesis.

In order to confirm a role for NOD1 *in vivo, H. pylori* infection in wild-type and NOD1 knock-out (KO) mice was investigated (experimental details in Boughan *et al*, 2006). Expression of murine β-defensin 4 (mBD4) a mouse orthologue of hBD2 was assessed (Schutte *et al.*, 2002, Semple *et al.*, 2005) from stomachs of infected mice. Seven days after infection, expression of mBD4 was clearly induced in the stomach of wild-type mice when compared to uninfected mice (Figure 4.14). In contrast, the expression of mBD4 was impaired in the stomach of NOD1 KO after *H. pylori* infection and values were comparable to uninfected mice. As a control, expression of mBD2 (another member of the β-defensin family) was also studied. Minimal mBD2 expression was noted in mouse stomach before and after *H. pylori* in wild-type and NOD1 KO mice when compared to ileal tissue (positive control), clearly indicating differential regulation of mBD2 and mBD4 in response to *H. pylori* infection (Figure 4.14). Interestingly, expression of macrophage inflammatory protein (MIP)-2, a functional analogue of the human interleukin 8, was more than twice as high in NOD1 KO mice than wild-type mice after exposure to *H. pylori* (Figure 4.14).

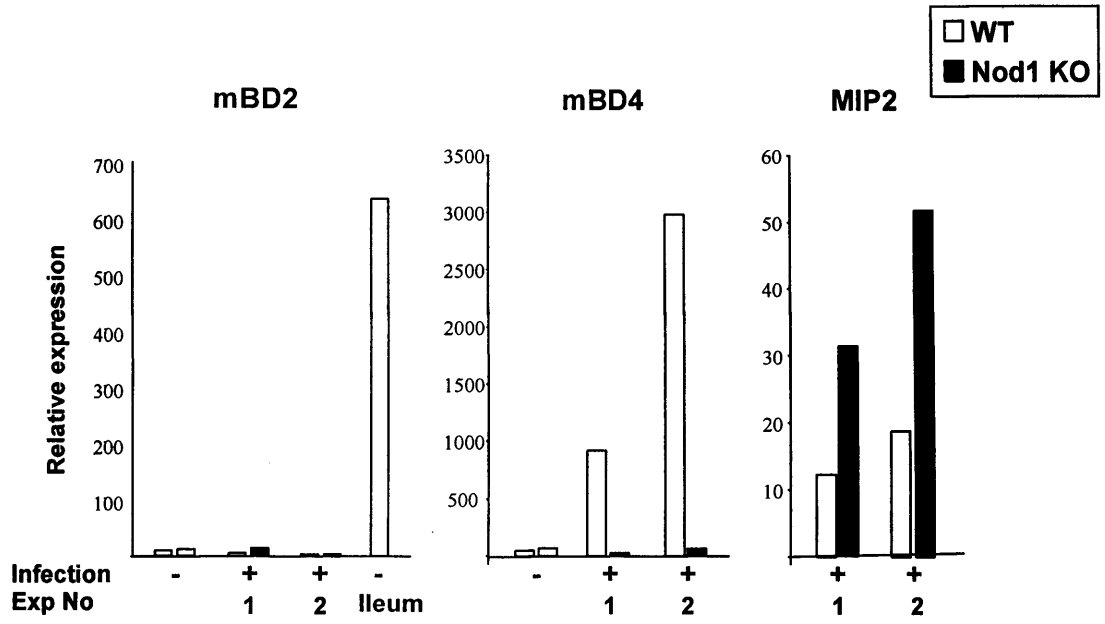


Figure 4.14: Impaired murine β-defensin 4 (mBD4) expression in NOD1-deficient mice after *H. pylori* infection.

Mice were inoculated intragastrically with *H. pylori* or PBS (uninfected) and sacrificed 7 days post-infection. Results denote expression levels of indicated genes in the stomach tissue after normalisation to *β*-actin as determined by quantitative Real-time PCR. 1 and 2 represent experiment number; each derived from different mice and performed independently. Ileum denotes expression analysis in ileal tissue from a mouse.

4.7 Conclusions

In the present study, a critical role for NOD1 in *H. pylori*-mediated NF-κB activation and hBD2 gene expression was established. In contrast, this pathway seems to play no significant role in hBD3 expression. This investigation is the first to highlight such potential differential regulation of hBD2 and hBD3 in any model of infection studied to date.

Although the transcription factor NF-κB has been implicated in both IL-1β and *H. pylori*-mediated hBD2 gene expression, very little is known about the regulation of hBD3 and this study is the first to investigate the regulatory mechanisms involved in hBD3 expression during an infection model. A minimal role for NF-κB in *H. pylori*-mediated hBD3 expression was found, which is not surprising in the context that no NF-κB binding sites have been identified in its promoter region (Wolk *et al.*, 2004).

Since molecular interactions leading to NF-κB activation and identity of upstream signalling events activated during *H. pylori* infection have remained unclear, the potential role of these transduction events in bacterial-mediated innate defence was explored in the present study. Firstly, time-dependent degradation of the inhibitory protein, IκBα, both upon stimulation with IL-1β and wild type *H. pylori* was sought. While IκBα is the major isoform of the inhibitory protein(s) studied by most researchers, NF-κB is known to be able to bind and reside as a complex with other members of this family (Whiteside *et al.*, 1997). No role for IκBβ protein in *H. pylori* infection was found. Western blotting is a semi-quantitative technique and these observations require confirmation by other methods. This series of experiments however, do suggest that *H. pylori* specifically activate NF-κB by removal of IκBα with IκBβ/NF-κB complexes remaining sequestered in the cytoplasm and playing a minimal role during infection. Unlike IκBα, IκBβ does not possess a nuclear export sequence (NES) and it has been proposed that this is required for newly synthesised

IκBα to transport NF-κB back into the cytoplasm from the nucleus (Rodriguez *et al.*, 1999), so this may help explain the difference in degradation pattern observed between the two inhibitory proteins.

Also, evidence suggests stimulus-induced phosphorylation of NF-κB p65 subunit, can play a key role in transcriptional activation (Zhong, 2002); the contribution of this regulatory mechanism in *H. pylori*-mediated activation was explored. *H. pylori*-infected AGS cells did not induce the phosphorylation of p65 subunit, whereas IL-1β stimulated cells phosphorylated p65 rapidly. It is possible that experimental time frame limited detection of phosphorylated p65 in *H. pylori* infected cells as time course was conducted for 4h. This data highlights differential NF-κB activation in the presence of the two stimuli tested and provides one possible explanation as to the more potent induction of hBD2 expression in the presence of cytokine when compared to bacterial-mediated increase.

Establishing that β-defensin gene expression is directly mediated by bacterial cross-talk and not due to indirect cytokine effect was an important finding. Dramatic inhibition of hBD2, hBD3 and IL-8 expression was observed in IL-1β stimulated cells in the presence of its antagonist; however, IL-1Ra had minimal effect on bacterial-mediated defensin expression. Also, minimal effect on IL-8 expression was noted in this study which reflects a previous study where the addition of an anti-IL1β antibody was unable to reduce *H. pylori*-mediated IL-8 levels (Nozawa *et al.*, 2002). In spite of this, it is likely that IL-1β paracrine effects following release from inflammatory cells would be an additional stimulus to epithelial defence gene expression *in vivo*. A study investigating hBD2 expression in response to *Escherichia coli* LPS in the skin epidermis showed weak hBD2 expression, which was further amplified by the production of IL-1β derived from monocytic cells which are present in the underlying tissue (Liu *et al.*, 2003). The present study was confined to

elucidating bacterial-epithelial cross-talk only and future studies should address the role and impact of other immune cells on bacterial-mediated defensin expression.

Studies performed in the presence of A46R and A52R showed marked inhibition of IL-1β-induced hBD2 expression. However, the presence of A46R or A52R proteins had no effect on bacterial-mediated hBD2 expression. Taken together, these findings provide evidence to exclude both IL-1β and TLR signalling in mediating *H. pylori*-induced hBD2 expression.

A significant role for NOD1 in the detection of *H. pylori* by epithelial cells has been reported (Viala *et al.*, 2004). Significant reduction of hBD2 but not hBD3 promoter activity in NOD1 siRNA transfected cells when exposed to *H. pylori* was observed, implicating NOD1 engagement in NF-κB mediated hBD2 expression. Furthermore, *in vivo* experiments investigating mBD4 (hBD2 orthologue) expression revealed that NOD1 KO mice exhibited impaired expression compared to wild type mice. The finding that MIP-2 was much higher in NOD1 KO mice than wild-type mice after exposure to *H. pylori* may be explained by increased *H. pylori* colonisation in the stomach of NOD1 KO mice and also highlights that unlike mBD4, MIP-2 gene expression is not exclusively dependent upon NOD activation (Viala *et al.*, 2004).

A role for NOD2 in GI epithelial cross-talk with microbes is in its infancy. No expression of NOD2 in gastric AGS cells and no up-regulation was observed during infection. Interestingly, a very recent study has demonstrated that functional binding sites for NF-κB in the hBD2 promoter are required for NOD2-mediated induction of hBD2 through its ligand muramyl dipeptide (MDP) in primary keratinocytes (Voss *et al.*, 2006). Together, these data suggest that NOD1 and NOD2 serve as intracellular pattern recognition receptors to enhance epithelial host defence by inducing the production of antimicrobial peptides such as hBD2 in response to infection.

In conclusion, NOD1 engagement is a major pathway triggered in gastric epithelia upon *H. pylori* infection. NOD1-driven NF-κB activation seems to regulate the expression of a subset of innate defence genes that include IL-8 and hBD2 but not hBD3.

CHAPTER 5

Role of *H. pylori*-mediated MAPK activation in β-defensin gene regulation

5.0 Background

In addition to the ubiquitous NF-κB pathway, mitogen activated protein (MAP) kinase signal transduction pathways are also activated by stimuli that play a crucial role in regulation of immune-mediated inflammatory processes (Cuschieri and Maier, 2005, Hommes *et al.,* 2003, Karin, 2004, Zhang and Dong, 2005). MAP kinases are serine/threonine kinases and comprise a three component, evolutionarily conserved, signalling cascade whereby a MAP 3-kinase phosphorylates a MAP 2-kinase, which in turn phosphorylates and activates a MAP kinase (Dong *et al.*, 2002). The three main family members are extracellular signal-regulated protein kinases (ERK1/2) primarily activated by growth factors; c-jun amino terminal kinase (JNK) and p38 MAP kinase, which respond to the presence of growth factors but are preferentially activated to cellular stress and cytokine stimulation. For example, the most studied MAP 3-Kinase, Raf-1 activates MEK1/2 which in turn phosphorylates ERK1/2, however, investigations into the regulation of MAP 3-Kinases have proven to be very complex (Symons *et al.*, 2006) as added cross-talk especially of upstream mediators means delineation of MAPK regulation in innate immune responses is likely to be equally complex.

The end result of MAPK signalling cascades is the activation of several transcription factors including activating transcription factor 2, ATF-2; Elk-1 and activating protein 1, AP-1. All three MAPK are known to activate the AP-1 family of transcription factors, which include the proto-oncogenes c-Jun, c-Fos and ATF-2 (Hommes *et al.*, 2003). AP-1 activates transcription of a number of target genes in growth-factor stimulated cells, and dysregulation of AP-1 has been implicated in abnormal cell growth leading to cancer (Meyer-ter-Vehn *et al.*, 2000, Mitsuno *et al.*, 2001).

Activation of transcription factor c-fos/c-jun (AP-1 complex) *via* the MAP kinase pathways during *H. pylori* infection has been well studied (see Figure 5.1; Keates *et al.*, 1999, Meyer-

ter-Vehn *et al.*, 2000, Mitsuno *et al.*, 2001, Naumann *et al.*, 1999, Naumann and Crabtree, 2004, Torok *et al.*, 2005).

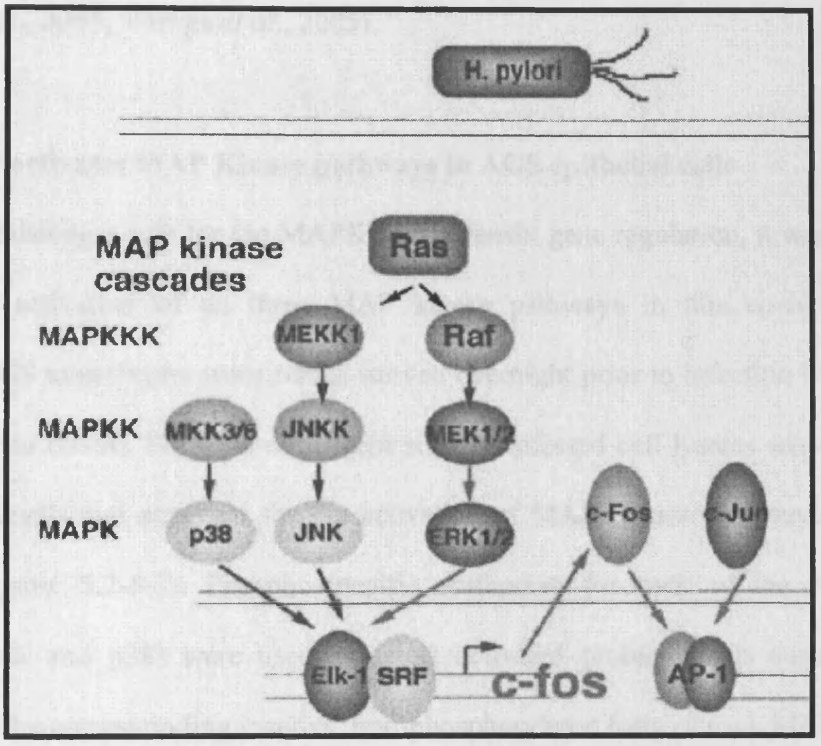


Figure 5.1 *H. pylori* activates MAPK signal transduction cascades (taken from Meyer-ter-vehn *et al*, 2000).

Regulation of MAP Kinase-mediated β-defensin during gastrointestinal (GI) infection and inflammation

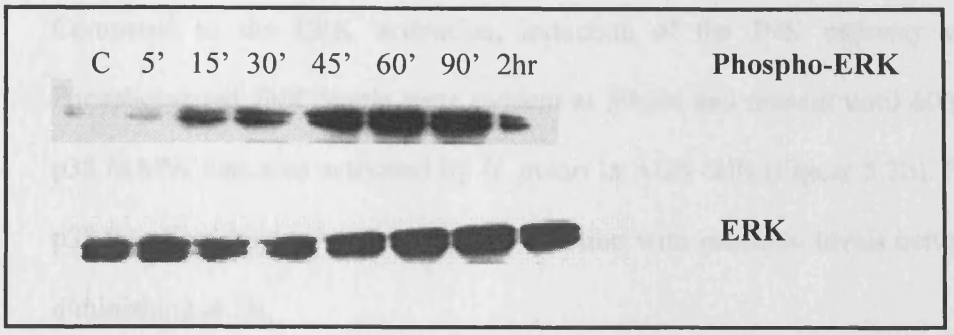
In addition to the three NF-κB bindings sites, there is one AP-1 binding site in the hBD2 promoter (Figure 4.2) and mutation in this AP-1 site has diminished reporter activity in response to several PAMPs suggesting involvement of AP-1 in eliciting and regulating hBD2 expression (Ogushi *et al.*, 2004, Vora *et al.*, 2004, Wehkamp *et al.*, 2004, Wehkamp *et al.*, 2006).

hBD3 and -4 are regulated by distinct NF-κB independent mechanism(s) and so far there are limited studies exploring these alternate pathways (Abiko *et al.*, 2003, Buhimschi *et al.*, 2004, Fahlgren *et al.*, 2004, Ganz, 2003, Harder *et al.*, 2004, Niyonsaba *et al.*, 2005, Sorensen *et al.*, 2005, Varoga *et al.*, 2005).

5.1 *H. pylori* activates MAP Kinase pathways in AGS epithelial cells

Prior to establishing a role for the MAPK in β-defensin gene regulation, it was pertinent to confirm the activation of all three MAP kinase pathways in this co-culture system. Confluent AGS monolayers were serum-starved overnight prior to infection with wild type *H. pylori* strain 60190. For time-dependent studies, infected cell lysates were collected at selective intervals and analysed for the activation of MAP Kinase pathways by Western blotting (Figures 5.2-5.3). Phospho-specific antibodies for each of the MAP Kinase (ERK1/2, JNK and p38) were used to detect activated protein levels during infection. Detection of the corresponding inactive, non-phosphorylated form of each MAP kinase was used as an internal control. *H. pylori* infection led to the activation of all three major MAP Kinases (Figures 5.2 and 5.3). Co-infection of AGS cells by two different cytotoxic (60190, 84-183) strains led to rapid induction of the ERK pathway (Figure 5.2a and b). Two immunoreactive bands p44 (ERK1, upper band) and p42 (ERK2, lower band) were first detected as early as 15min post-infection with maximal levels achieved by 60min and levels remained elevated up to 2h. IL1β-stimulated cells were investigated in parallel (positive control) and a similar pattern of rapid activation was observed (Figure 5.2c). Kinetically, IL-1β stimulation was more rapid with maximal levels attained by 30min, and complete inactivation 60min post stimulation. In contrast, a more sustained activation was observed in the presence of both strains of bacteria (Figure 5.2a and b compared to c). Detection of two immunoreactive bands, representing phosphorylated p54 (upper band) and

(a)



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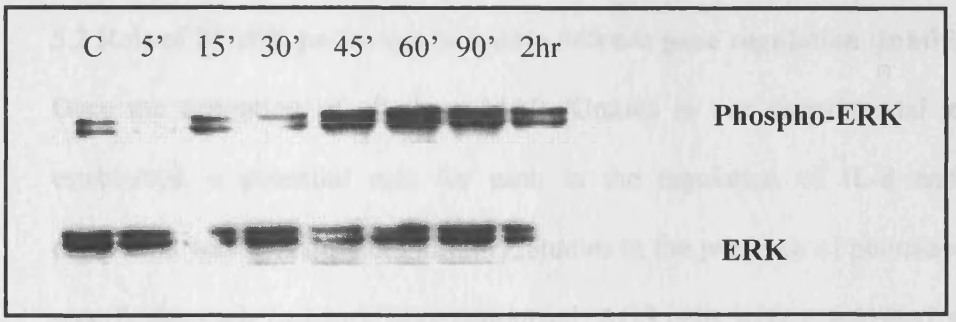

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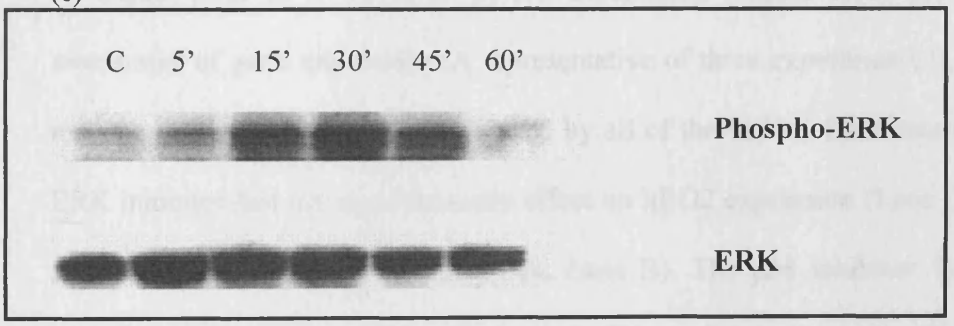


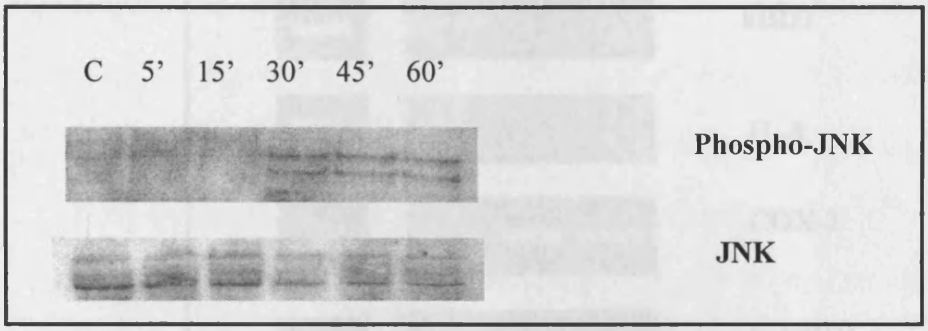
Figure 5.2 Activation of the ERK MAP kinase pathway. AGS cells were either co-cultured with *H. pylori* (a) 60190, (b) 26695 ($1x10^8$cfu/ml) or treated with (c) IL-1β (20ng/ml) for up to 2h. Cell lysates were prepared and subjected to SDS-PAGE followed by western blotting with phospho-specific antibody for ERK1 (p44 upper band) and ERK2 (p42 lower band). Blots were stripped and re-probed with non-phospho antibody specific for ERK MAPK for equal protein loading. The blots shown are representative of three individual experiments.

p46 (lower band) were indicative of activation of the JNK MAPK pathway (Figure 5.3a). Compared to the ERK activation, induction of the JNK pathway was very modest. Phosphorylated JNK levels were evident at 30min and present until 60min post infection. p38 MAPK was also activated by *H. pylori* in AGS cells (Figure 5.3b). Phosphorylation of p38 was first detected at 45min post-infection with maximal levels between 60 and 90min diminishing at 2h.

5.2 Role of MAPK pathways in innate defence gene regulation (inhibitor studies)

Once the activation of all three MAP Kinases in our experimental model system was established, a potential role for each in the regulation of IL-8 and β-defensin gene expression was investigated. Initially, studies in the presence of pharmacological inhibitors specific for each pathway were conducted. AGS cells were pre-treated with each inhibitor for 30min prior to IL-1β or *H. pylori*- stimulation (Figure 5.4a). RT-PCR allowed the assessment of gene expression. A representative of three experiments is shown. *H. pylori*-mediated hBD2 expression was reduced by all of the MAPK inhibitors (Figure 5.4a). The ERK inhibitor had the most dramatic effect on hBD2 expression (Lane C) followed by the JNK inhibitor, SP100625 (Figure 5.4a; Lane B). The p38 inhibitor, however, was least effective in decreasing hBD2 mRNA levels (Figure 5.4a; Lanes A). Inhibition of all three MAPK pathways showed a significant reduction in IL-8 gene expression. Interestingly, induction of Cox-2 (an innate defence gene) mRNA was observed in the presence of JNK and ERK inhibitors (Lanes B and C). It is likely that this effect is due to increased stability of the Cox-2 mRNA. Figure 5.4b represents the effects of MAPK inhibitors on IL-1β stimulated innate immune gene expression. The p38 inhibitor had an inhibitory effect on hBD2 expression (Lane A). In contrast the ERK inhibitors, PD98059 and U0126 were less effective in causing a reduction of hBD2 defensin expression (Lanes B and C). Complete

(a)



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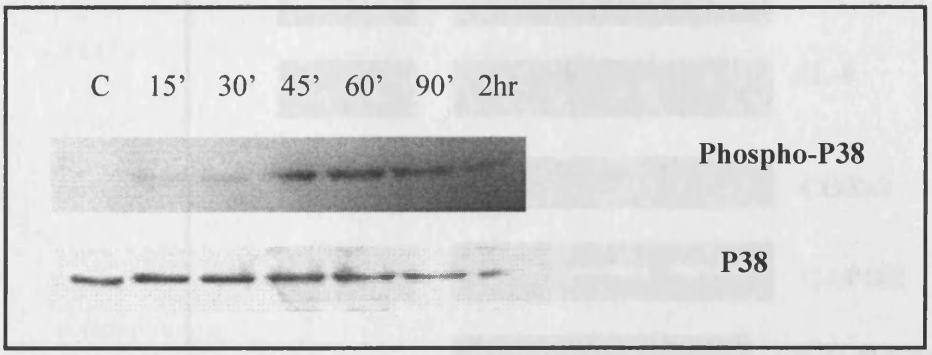


Figure 5.3 Slow and modest induction of JNK and p38 MAPK pathways by *H. pylori*. *H. pylori* strain 60190 was used to infect AGS monolayers and cell lysates were subjected to Western Blot analyses using phospho-specific antibodies for (a) JNK p54 (upper band) and p46 (lower band), and (b) p38 MAPKs.

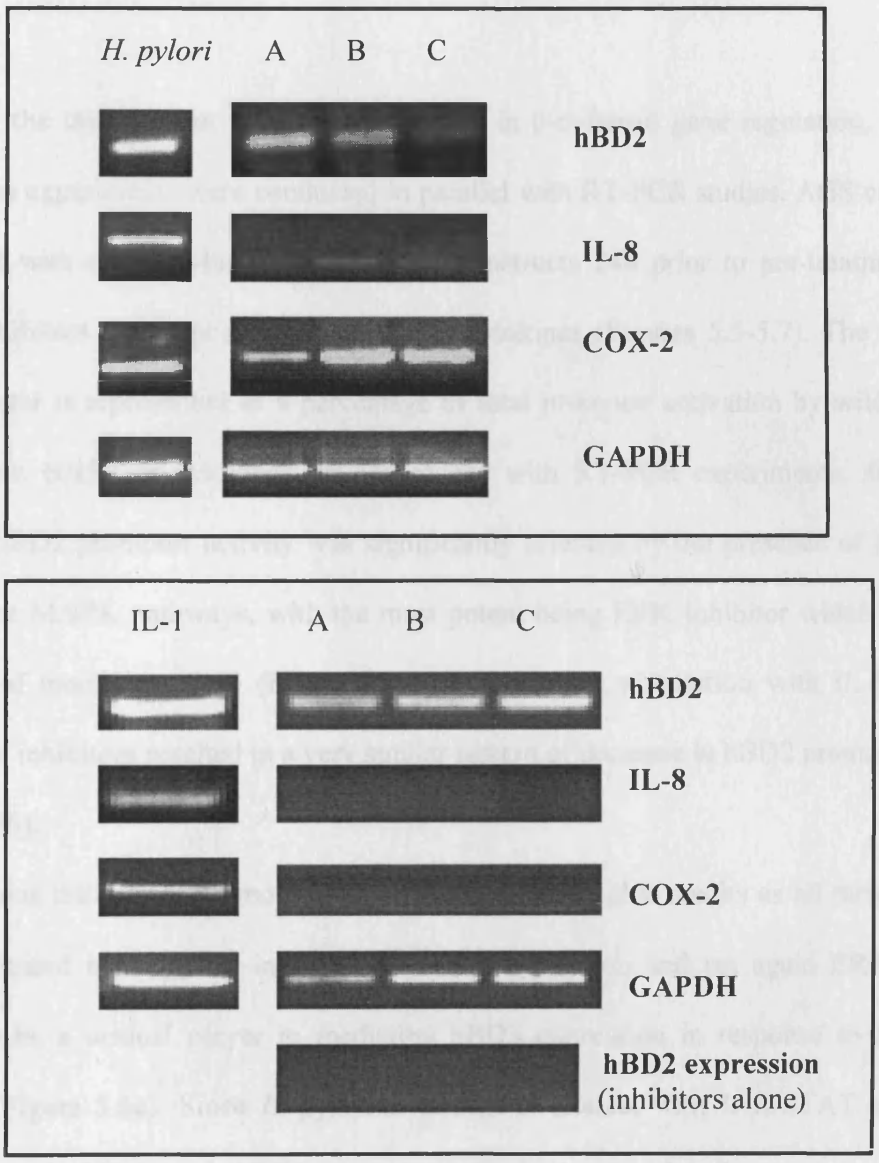


Figure 5.4: Effect of MAPK inhibitors on (a) *H. pylori*- and (b) IL-1β-induced gene expression.

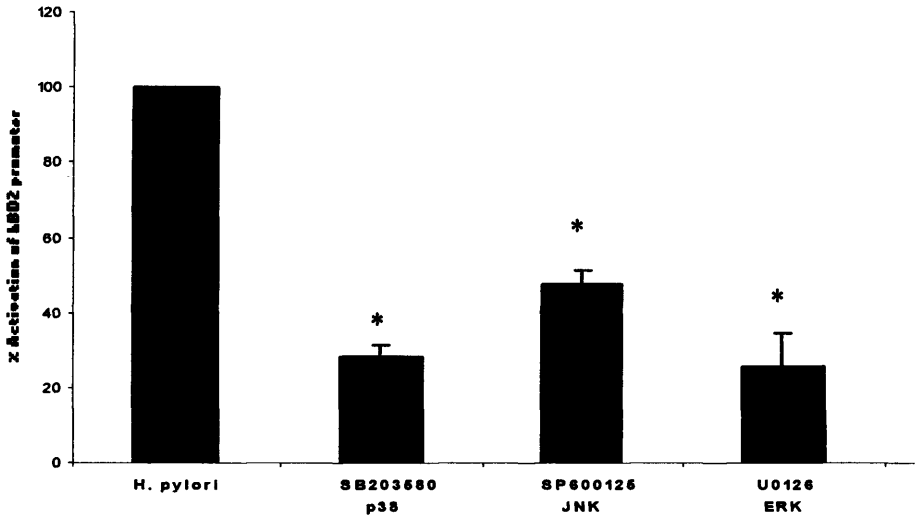
AGS cells were pre-treated with pharmacological inhibitors for each MAPK pathway prior to stimulation. (a) Lane A represents cells treated with bacteria+SB 203580, Lane B, bacteria+SP 600125; Lane C, bacteria+ERK inhibitor PD 98059, treated cells. (b) Lane A: IL-1β+SB 203580; Lane B and C, IL-1β+ERK inhibitors, PD 98059 and U0126.

abrogation of IL-8 and Cox-2 mRNA was observed in the presence of p38 and ERK inhibitors.

To clarify the involvement of particular MAPK in β-defensin gene regulation, transient transfection experiments were conducted in parallel with RT-PCR studies. AGS cells were transfected with defensin-luciferase promoter constructs 24h prior to pre-treatment with MAPK inhibitors and exposure to bacteria or cytokines (Figures 5.5-5.7). The effect of each inhibitor is represented as a percentage of total promoter activation by wild type *H. pylori* strain 60190 or cytokines. In accordance with RT-PCR experiments, *H. pylori*-mediated hBD2 promoter activity was significantly affected by the presence of inhibitors for all three MAPK pathways, with the most potent being ERK inhibitor which caused a reduction of more than 70% (Figure 5.5a). Interestingly, stimulation with IL-1β in the presence of inhibitors resulted in a very similar pattern of decrease in hBD2 promoter levels (Figure 5.5b).

Investigations into hBD3 promoter activity yielded comparable results as all three MAPK were implicated in *H. pylori* induction of hBD3 expression and yet again ERK MAPK seemed to be a critical player in mediating hBD3 expression in response to *H. pylori* infection (Figure 5.6a). Since *H. pylori* is known to interact with JAK/STAT signalling (Mitchell *et al.*, 2004), and hBD3 promoter analysis has revealed the presence of STAT binding sites (Wolk *et al.*, 2004), JAK inhibitor was also added to the co-culture system to determine a role for JAK/STAT signalling in *H. pylori*-mediated hBD3 expression. The presence of the JAK inhibitor induced a significant reduction in hBD3 (Figure 5.5a) promoter activity which was comparable to the effects observed in the presence of U0126. As IFNγ is a known agonist for hBD3, it was used as a positive control and inhibitor studies exposed roles for only ERK and JNK but not p38 in cytokine-mediated promoter activity (Figure 5.6b).

(a)



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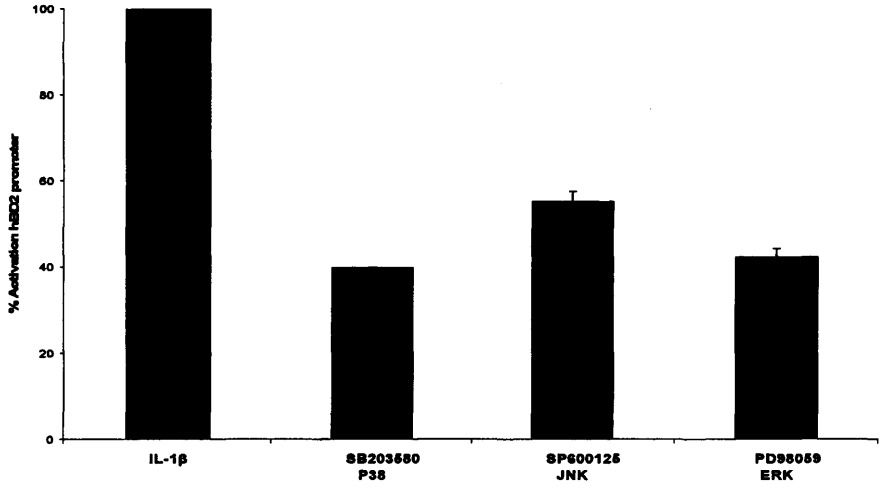
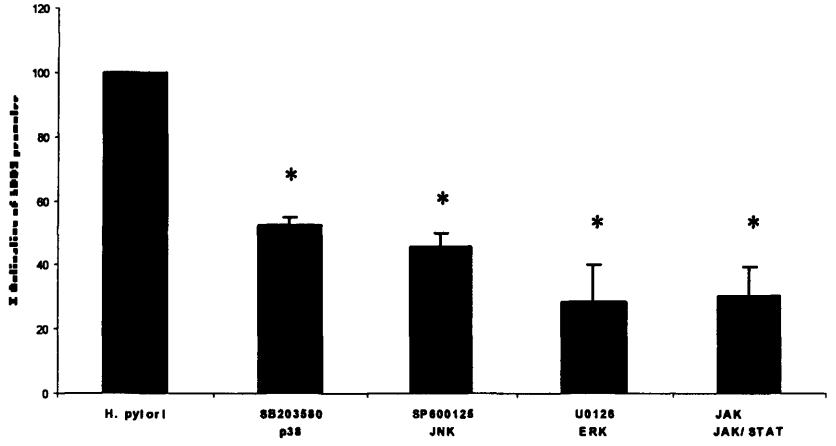


Figure 5.5: Potential role of MAP Kinase pathways in differential hBD2 regulation.

hBD2 promoter transfected AGS cells were pre-treated with specific inhibitors (p38; SB203580 25 μM, JNK; SP100625 50 μM, and ERK; U0126/PD98059 25 μM) and promoter activity quantified 8h post-stimulation. Results are presented as percentage activation of hBD2 promoter activity compared to (a) bacterial infection or (b) IL-1β stimulation alone.

(a)



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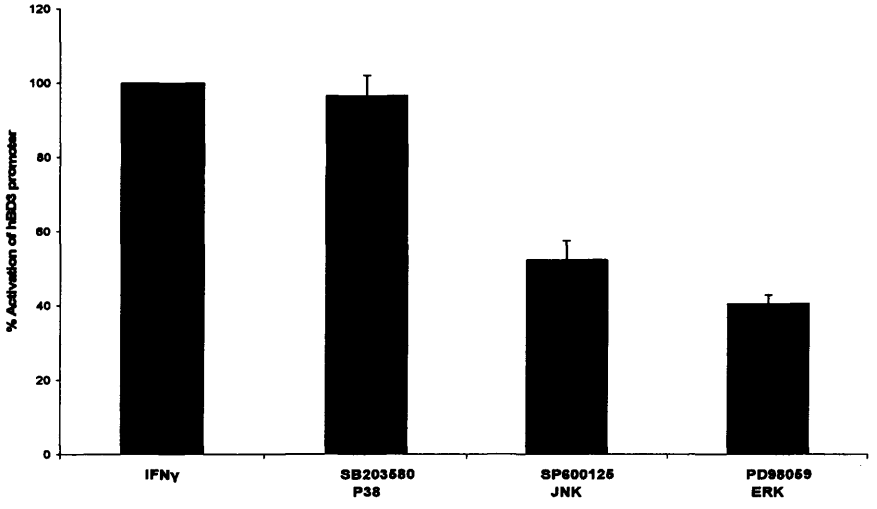


Figure 5.6: Potential role of MAP Kinase pathways in differential hBD3 regulation.

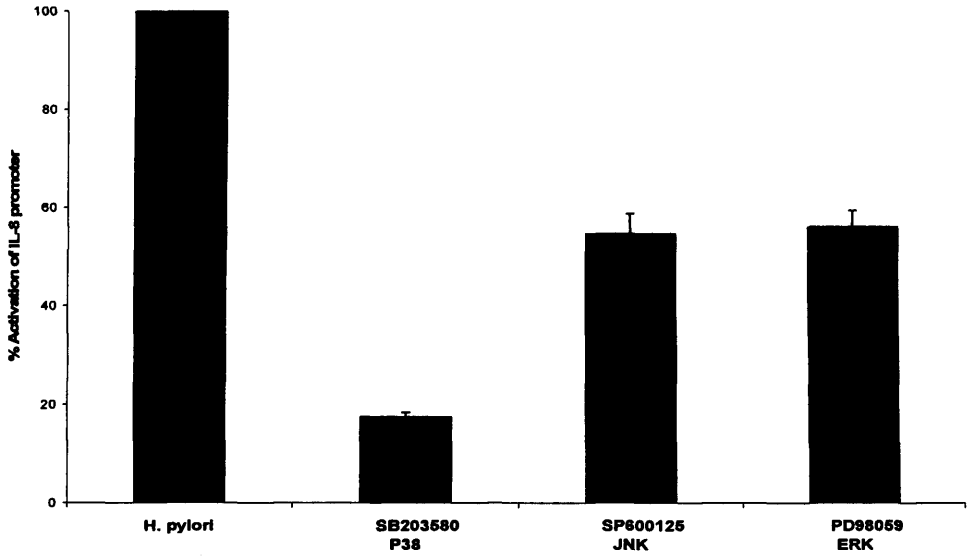
hBD3 promoter transfected AGS cells were pre-treated with specific inhibitors and exposed to (a) *H. pylori* infection or (b) IFNγ stimulation. Results are presented as percentage activation of hBD3 promoter activity compared to (a) bacterial infection or (b) IFNγ stimulation alone. Values are expressed as mean ±SEM from a representative of three experiments conducted in triplicates.

In order to confirm IL-8 gene expression observed in RT-PCR findings in response to *H. pylori*, IL-8 promoter function was also assessed by luciferase-promoter assays. All three MAPK were involved in *H. pylori* -mediated IL-8 promoter regulation, but most prominent was the role of p38 where nearly 80% decrease in expression was detected in the presence of the inhibitor (Figure 5.7a). Both JNK and ERK inhibitors had very similar effects in reducing IL-8 promoter activity (more than 40% inhibition). Interestingly, the promoter activity observed in the presence of the different inhibitors during *H. pylori* infection was mirrored when cells were treated with IL-1β (Figure 5.7b), suggesting the involvement of all three pathways in both bacterial and cytokine-induced IL-8 expression. JNK and p38 inhibitors reduced hBD2 and IL-8 expression levels to a similar magnitude whereas there was more of a contribution of the ERK pathway in mediating hBD2 rather than IL-8 expression. RT-PCR findings suggest ERK is involved in *H. pylori*-induced hBD2 up-regulation whereas promoter analyses consistently implicated ERK MAPK pathway more than p38 and JNK in both hBD2 and hBD3 cytokine and *H. pylori* –mediated expression.

5.3 Activation of discrete MAP kinase pathways leads to differential β-defensin expression ,

Although the use of pharmacological inhibitors can estimate the potential role of each individual pathway, the complexity of cross-talk between various signal transduction events makes firm conclusions difficult. To gain better understanding of the exact contribution of individual MAP kinase pathways to β-defensin gene expression, two conditional kinases that stimulate defined MAP kinase pathways were utilised (Garner *et al.*, 2002, Todd *et al.*, 2004). HR1 cells are HEK293 cells expressing ΔRaf-1:ER* which specifically activates the ERK pathway following stimulation with 4-Hydroxytamoxifen (4-HT) (Figure 5.8) whereas HM3 cells are HEK293 expressing ΔMEKK3:ER*, which leads to strong

(a)



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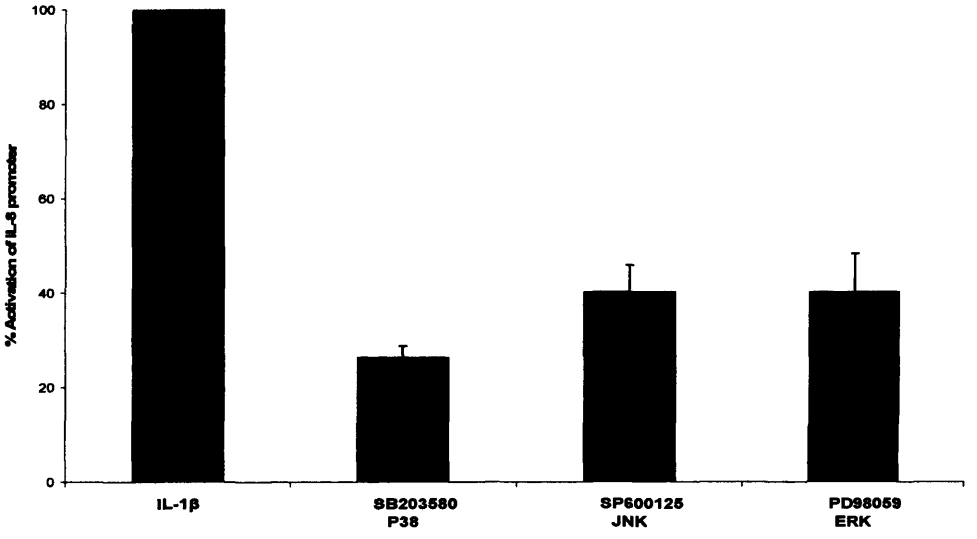

Figure 5.7: Potential role of MAP Kinase pathways in IL-8 regulation.

(a) *H. pylori*- and (b) IL-1β- mediated IL-8 promoter activity was assessed in the presence of MAPK inhibitors. Results are presented as percentage inhibition of promoter activity compared to bacterial infection alone. Values are expressed as mean ±SEM from a representative of three experiments conducted in triplicates.

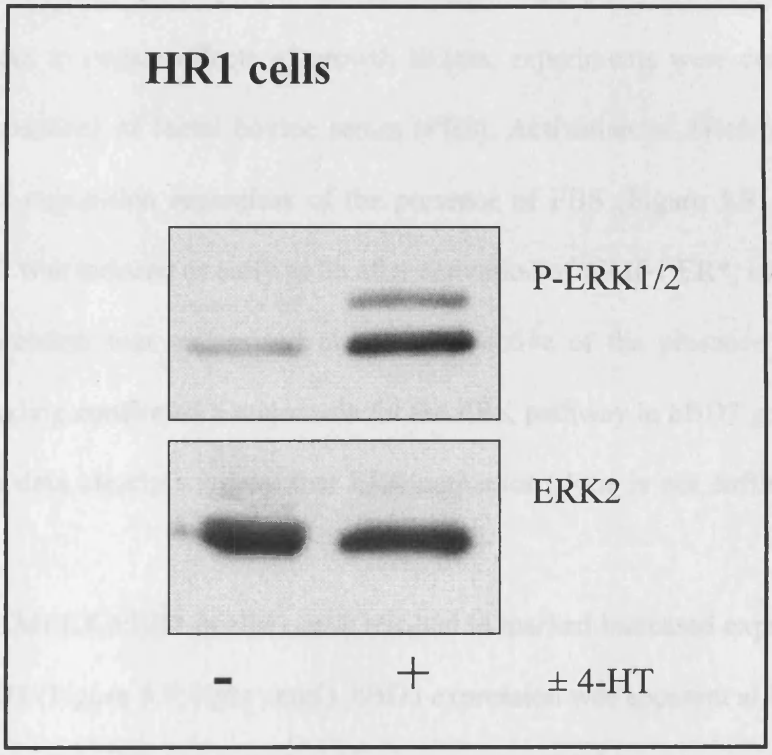

Figure 5.8: Activation of ERK MAPK pathway.

HR1 epithelial cells expressing ΔRaf-1:ER* were stimulated with 4-Hydroxytamoxifen (4-HT; 100nM) and cell lysates were subjected to SDS-PAGE followed by Western Blotting with a phosho-specific ERK antibody. Specific activation of the ERK pathway was observed with cells treated with 4-HT compared to untreated controls. Kindly performed by K. Ewings, Babraham Institute, Cambridge.

activation of the endogenous p38 and JNK pathways upon stimulation with 4-HT and a weaker activation of ERK (Todd *et al.*, 2004). HR1 or HM3 cells were treated with 4-HT (100nM) for 8 or 24h prior to assessment of gene expression by RT-PCR to allow identification of pathways implicated in β-defensin expression in the absence of any stimuli. In order to negate effects of growth factors, experiments were conducted in the absence (or presence) of foetal bovine serum (FBS). Activation of ΔRaf-1:ER* failed to increase hBD2 expression regardless of the presence of FBS (Figure 5.9; left panel). In contrast, hBD3 was induced as early as 8h after activation of ΔRaf-1:ER*, in the absence of FBS, and expression was maintained at 24h irrespective of the presence or absence of serum. This finding confirmed a major role for the ERK pathway in hBD3 gene expression, in contrast the data clearly suggests that ERK activation alone is not sufficient to induce hBD2.

Activation of ΔMEKK3:ER* in HM3 cells resulted in marked increased expression of both hBD2 and hBD3 (Figure 5.9; right panel). hBD3 expression was apparent at 8h with further increase noted at 24h. The magnitude of response was slightly weaker in the absence of FBS. In contrast, induction of hBD2 expression was slower as it was only observed at 24h with weaker induction in the absence of FBS. The greater expression of hBD2 and hBD3 by ΔMEKK3:ER* compared to ΔRaf-1ER* cells raised the possibility that p38 and/or JNK may also contribute to the up-regulation of β-defensins.

The role of ERK pathway in hBD3 expression was further highlighted utilising U0126 which reduced hBD3 levels to those observed in control unstimulated cells (Figure 5.10; left panel). These experiments suggest unequivocally that the ERK pathway is both necessary and sufficient for hBD3 gene expression in the absence of other signalling events. In contrast, the ERK pathway alone was unable to induce hBD2 expression.

To further dissect the contributions of p38 and JNK MAPK pathways in mediating

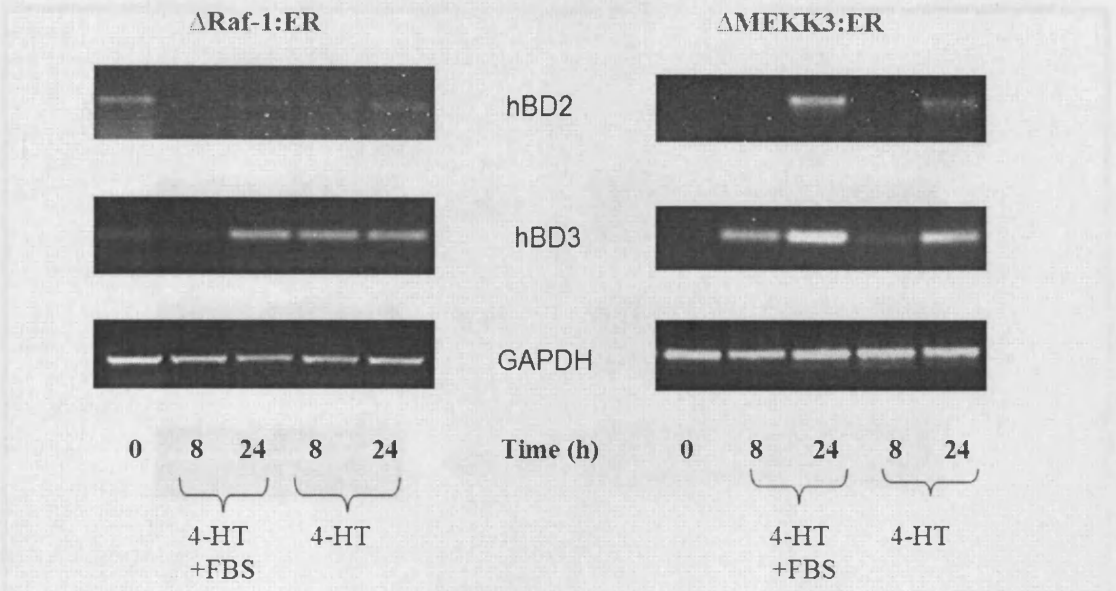

Figure 5.9 Activation of ERK MAPK pathway is sufficient for hBD3 but not hBD2 gene expression.

HR1 cells (expressing the ΔRaf-1:ER*, left panel) and HM3 cells (expressing ΔMEKK3:ER*, right panel) were stimulated with 4-HT for a period of 8h or 24h in the presence or absence of serum. β-defensin gene expression was evaluated by RT-PCR and a gel from a representative of three independent experiments is shown.

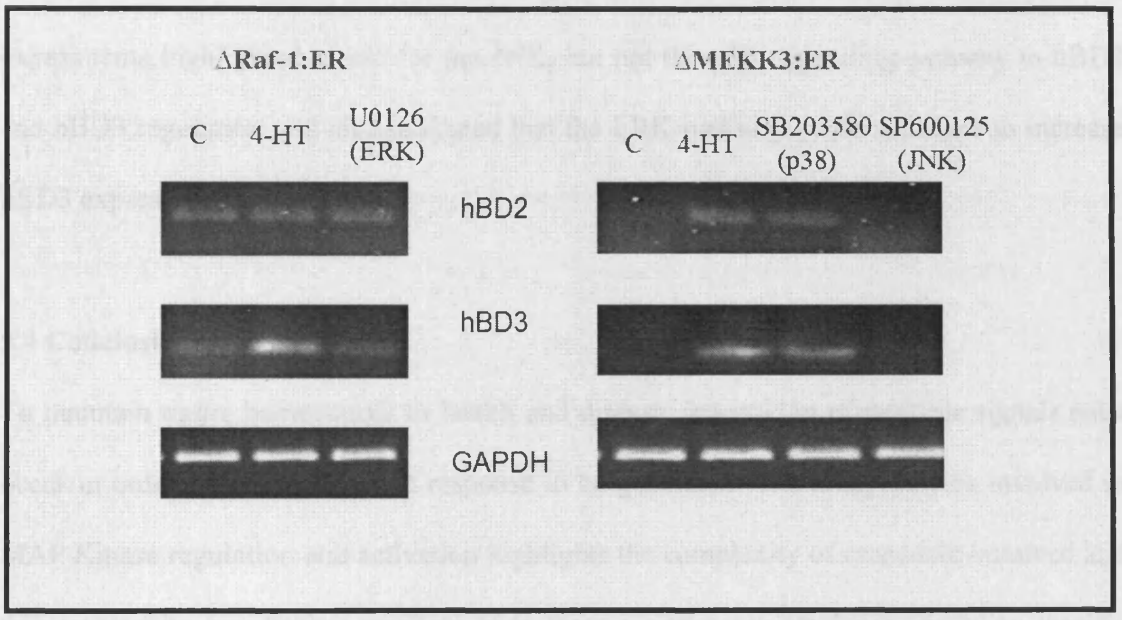

Figure 5.10 JNK MAPK is involved in regulation of both hBD2 and hBD3 expression. HR1 cells and HM3 cells were pre-treated with pharmacological inhibitors for ERK (U0126), p38 (SB203580) and JNK (SP600125) pathways for 1h prior to addition of 4-HT for 8h. Gene expression was assessed by RT-PCR. Treatment of HM3 cells with 4-HT + SP600125 (last lane; right panel) caused abrogation of hBD2 and hBD3 expression. A representative gel is shown.

defensin gene expression, inhibitors prior to and during activation by 4-HT were used. As shown in Figure 5.10 (right panel) the presence of the JNK inhibitor SP600125 completely abolished the induction of hBD2, whereas the p38 inhibitor SB203580 had little effect. This result was reflected to a similar degree in hBD3 expression. In summary, these experiments highlighted a role for the JNK, but not the p38, signalling pathway in hBD2 and hBD3 regulation and also indicated that the ERK pathway could act alone to increase hBD3 expression.

5.4 Conclusions

To maintain tissue homeostasis in health and disease, integration of multiple signals must occur in order for an appropriate response to be generated. The many players involved in MAP Kinase regulation and activation highlights the complexity of cross-talk involved and this complexity was further emphasised in these experiments employing pathway-specific inhibitors.

Use of pharmacological inhibitors implicated all three pathways to varying degrees in both hBD2 and hBD3 gene regulation. RT-PCR analyses for hBD2 suggested ERK is required for induction when cells were exposed to *H. pylori* and transfection studies indicated that ERK pathway seemed to play a role in both cytokine and bacterial-mediated β-defensin expression. Additionally, regulation of the host innate immune response upon challenge with *H. pylori* as a broad response was studied; hence expression of IL-8 and Cox-2 was investigated as both are critical genes implicated in *H. pylori* disease. IL-1β and *H. pylori*-mediated IL-8 expression was diminished by JNK, p38 and ERK inhibitors, in contrast, cytokine stimulation of COX-2 was abrogated by p38 and ERK inhibitors whereas bacterial stimulation was only lessened by the p38 inhibitor. This data indicates there are discrete

signalling mechanisms which are stimuli-specific involved in the regulation of this subset of innate immune genes.

The inclusion of the JAK inhibitor revealed a role for the JAK-STAT signalling pathway in *H. pylori*-mediated hBD3 regulation. This finding correlates with the presence of STAT binding sites in the hBD3 promoter (Wolk *et al.*, 2004) and IFNγ, which typically signals via JAK-STAT pathways, being a potent agonist of hBD3. IFNγ production is characteristic of a Th1 immune response observed during *H. pylori* infection and a recent study has documented the presence of the bacterium caused a reduction in IFNγ-induced STAT1 DNA binding (Mitchell *et al.*, 2004). This result was independent of the *cagA*, *cagE*, and *vacA* status of the infecting *H. pylori* strain and these findings taken together with the involvement of JAK-STAT signalling in hBD3 expression may represent a mechanism by which the bacterium can modulate/decrease host hBD3 antimicrobial response, thus aiding persistent colonisation.

HR1 and HM3 cells were employed to single out MAPK pathways in β-defensin regulation as any firm conclusions from the pharmacological studies were not made. This novel strategy allowed us to selectively activate individual MAPK pathways in the absence of any other signalling event. The most contrasting data between the inhibitor studies and the conditional mutants was obtained for hBD2 gene expression as the use of U0126 suggested ERK activation to be involved in both hBD2 and 3 gene expression (Figures 5.5 and 5.6) however, ERK activation alone was unable to induce hBD2 expression (Figure 5.9). *This important finding suggests that ERK pathway must synergise or cross-talk with other pathways to modulate hBD2 expression during H. pylori infection.* In contrast, ERK pathway alone was sufficient to induce hBD3 expression. Addition of specific inhibitors in HM3 cell system revealed a significant role for the JNK pathway in β-defensin expression, whereas p38 was found to be unnecessary. The finding implicating JNK but not p38 in

induction of β-defensins represents another discrepancy between the results obtained for hBD2 expression with the MAPK inhibitors in AGS cells and the use of the conditional kinase HEK 293 cell line, suggesting that unlike the simple and limited pathway(s) in the HEK 293 system, the presence of infection is likely to initiate many overlapping signalling pathways involved in molecular cross-talk.

Unlike NF-κB activation, it is unclear whether activation of the MAPK by *H. pylori* is dependent upon an intact *Cag*PAI, as one study has documented induction of MAPK pathways by *Cag*PAI negative strains but to a lesser extent that wild type strains (Keates *et al*, 1999). Another study showed that strains specifically lacking CagA or strains that are mutated in *cag* genes encoded by the *Cag*PAI, do not induce MAP kinase activity (Meyer-ter-Vehn *et al*, 2000). Such evidence implies that modulation of host epithelial cell signal transduction responses may contribute to pathogenesis of disease (Peek, 2001).

A similar dependency for JNK but not the p38 or ERK pathway in hBD2 expression has also been observed during *E.coli Nissle* 1917-mediated infection of intestinal epithelial cells (Wehkamp *et al.*, 2004). Conversely, *Pseudomonas aeruginosa*-mediated hBD2 induction in human keratinocytes was blocked by treatment of NF-κB, JNK and p38 inhibitors but not ERK inhibitors (Wehkamp *et al.*, 2006).

Previous studies in oral epithelia with the periodontal bacterium *Fusobacterium nucleatum*, have indicated that the MAP kinases are involved in hBD2 regulation but NF-κB is not (Krisanaprakornkit *et al*, 2002). Further to this, hBD2 induction by both commensal and pathogenic bacteria was partially or completely blocked by inhibitors of the JNK and p38 pathways whereas only hBD2 up-regulation by pathogenic strains was blocked by inhibitors of NF-κB in both oral and foreskin keratinocytes (Chung and Dale, 2004). These findings suggest that epithelial cells share common signalling mechanisms to distinguish between the distinct pathways utilised by commensal and pathogenic bacteria. Collectively,

the results from all these investigations highlight the differential signalling pathways employed by different bacterial species at distinct anatomical sites.

CHAPTER 6

A Potential Role for *H. pylori*–induced EGF Receptor Transactivation in hBD3 Expression

6.0 Background

The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases and plays a critical role in mediating the regulation of cell proliferation, survival and wound healing (Stein and Staros, 2000). Binding of a growth factor ligand to its receptor initiates a cascade of phosphorylation events orchestrated by protein kinases, ultimately leading to activation of transcription factors and changes in gene expression. EGFR is a 170kDa cell surface glycoprotein present in many cell types, its structure comprises three main regions; i) a glycosylated extracellular ligand-binding domain, ii) a hydrophobic transmembrane domain, and iii) a cytoplasmic conserved protein tyrosine kinase domain (Castillo *et al.*, 2004).

There are 11 ligands identified for this family of receptors to date including EGF, transforming growth factor-α (TGFα), HB-EGF (heparin-binding) and amphiregulin (Olayioye *et al.*, 2000) and many of these are expressed as membrane-bound proteins themselves. Upon proteolytic cleavage the ligands become available for receptor binding and are able to initiate downstream signalling events such as the involvement of the MAPK or PI3K/Akt pathways (Bazley and Gullick, 2005). The important role in regulating cellular growth and developmental processes is highlighted by the hyperactivation of EGFR leading to cancerous tumor growth. The loss of regulation of normal processes may occur via several different mechanism(s) such as overexpression of receptor, autocrine overproduction of ligands or EGFR mutations (Castillo *et al.*, 2004). Such mechanisms may result in increased cell growth and proliferation with a decrease in apoptosis.

Divergent signalling responses have been reported during infection with *cag*+ and/or *cag*- strains, particularly *cag*+ strains preferentially activating the MAPK, NF-κB and proto-oncogenes c-fos and c-jun (Glocker *et al.*, 1998, Keates *et al.*, 1999, Meyer-ter-Vehn *et al.*,

2000). The induction of genes involved in immunity, cell growth, proliferation and apoptosis may help to explain why individuals infected with *cag*+ strains are more likely to develop gastric cancer (Peek, Jr., 2002). Several studies have revealed transactivation of the epidermal growth factor receptor (EGFR) by *Helicobacter pylori* and in addition, gastric expression levels of EGFR ligands (EGF, HB-EGF) and EGFR itself are elevated (Fischer *et al.*, 2003, Keates *et al.*, 2001, Wallasch *et al.*, 2002). The relevance of the EGFR pathway in wound healing processes and mucosal repair makes it a particularly critical pathway for further investigation (Barnard *et al.*, 1995, Leahy, 2004).

6.1 Epidermal Growth Factor Receptor (EGFR) transactivation by *H. pylori*

The experiments so far have implicated NOD1 as the upstream mediator of NF-κB-induced hBD2 but not hBD3 gene expression (Chapter 4). The lack of NF-κB binding sites in the promoter region of hBD3 in addition to the NF-κB inhibitor studies ruled out a role for NF-κB in the regulation of hBD3. This prompted exploration into potential candidate upstream receptors in driving inducible hBD3 expression. The involvement of ERK MAPK in hBD3 but not hBD2 up-regulation (Chapter 5) led to the hypothesis that EGFR may be an upstream mediator of hBD3 expression since it is known to be an upstream signalling event of ERK MAPK.

Previous reports have demonstrated the ability of *H. pylori* to activate EGFR signal transduction cascades in gastric epithelial cells (Keates *et al.*, 2001, Pai *et al.*, 1998, Romano *et al.*, 1998, Wallasch *et al.*, 2002). In addition, gastric expression levels of EGFR ligands (EGF, HB-EGF and amphiregulin) and EGFR itself are elevated during infection both with *cag*+ and *cag*- strains (Keates *et al.*, 1999, Romano *et al.*, 1998, Wallasch *et al.*, 2002). Prior to investigating the role of EGFR in bacterial-mediated hBD3 expression, transactivation status of EGFR during *H. pylori* infection in this experimental model system

had to be established. Tyrosine phosphorylation of EGFR was followed by Western blotting utilising an antibody that recognises tyrosine phosphorylated proteins. Specificity of EGFR was confirmed by reprobing blots with an EGFR-specific antibody. Activation of EGFR was observed as early as 1h post-infection (Figure 6.1). Time-dependent phosphorylation of EGFR was followed up to 4h post-infection. Selective inhibition of EGFR was verified by addition of a neutralising antibody and pharmacological inhibitor (AG1478; 3μM). In the presence of inhibitory reagents tested, transactivation of EGFR was blocked, confirming specificity of reaction. Cells were also treated with 50ng/ml of recombinant EGF, included as a positive control.

6.2 Potential role of EGFR-activation in mediating β-defensin gene expression

Next, the role of EGFR activation in mediating β-defensin gene expression was examined. AGS cells were either left alone or pre-treated with several inhibitory reagents for EGFR for 30-60min prior to *H. pylori* infection. A neutralising antibody specific for EGFR and two EGFR-specific pharmacological inhibitors, leading to inhibition of subsequent downstream signalling events, the competitive inhibitor AG1478 or the irreversible inhibitor PD168393 were used. Figure 6.2 depicts EGFR signalling pathway and in particular the target of the two inhibitory reagents is highlighted.

Specific inhibition of EGFR either in the presence of the inhibitors or neutralising EGFR antibody resulted in significant reduction in hBD3 mRNA was found (Figure 6.3). The most effective inhibition was observed by the neutralising antibody (10μg/ml) where hBD3 gene expression was reduced by 60%. The two inhibitors were similarly potent in inhibiting hBD3 as expression levels were decreased by 40-50%. Interestingly, no effect of these agents was demonstratable on *H. pylori*-mediated hBD2 gene expression (Figure 6.3). As expected, these findings ruled out a role for EGFR in regulating hBD2 gene expression but

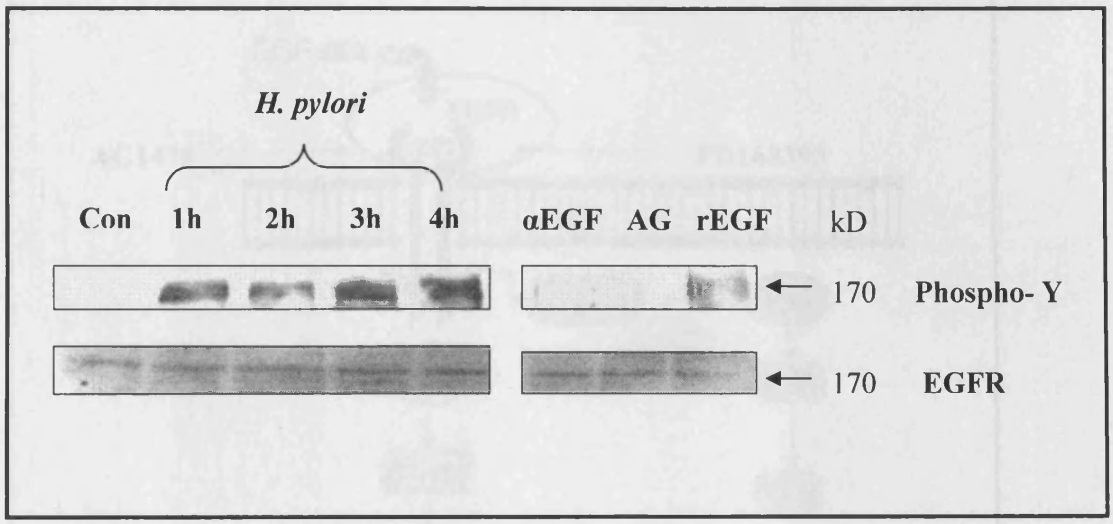

Figure 6.1: Time-dependent increase in EGFR tyrosine phosphorylation by *H. pylori*. AGS cells were infected with wild type *H. pylori* ($1x10^8$ cfu/ml) for up to 4h and cell lysates taken at hourly intervals were subjected to immuno-blotting. Tyrosine phosphorylation was followed with a phospho-specific antibody followed by EGFR specific antibody to confirm equal loading. Selective inhibition of EGFR phosphorylation was observed in the presence of AG1478 (AG), a specific inhibitor, and EGFR neutralising antibody (αEGF) 2h post-infection. Recombinant EGF (50ng/ml; 1h) was included as a positive control.

in contrast, provided the first clear indication for EGFR-mediated hBD3 regulation in our model of infection.

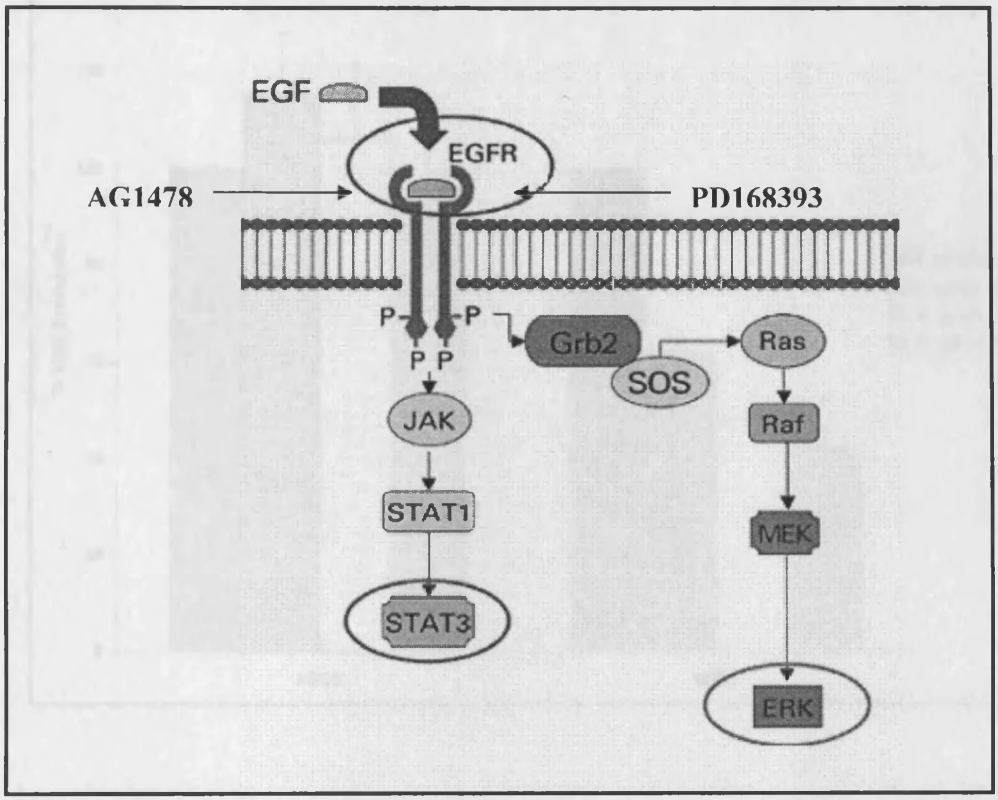

Figure 6.2 AG1478 and PD168393 target EGFR and downstream signalling cascades.

6.3 Potential role of EGFR-activation in mediating β-defensin peptide expression

In order to substantiate the gene expression findings, peptide analysis was conducted by Western Blotting. There was minimal peptide detected in control uninfected cells but both hBD2 and hBD3 peptides were induced upon infection with wild type *H. pylori* (Figure 6.4), 24h post-infection. The increase in hBD3 protein levels during infection was abrogated in the presence of AG1478, however, inhibition of EGFR pathway did not affect hBD2 peptide levels. Recombinant peptides were included as positive controls (Figure 6.4).

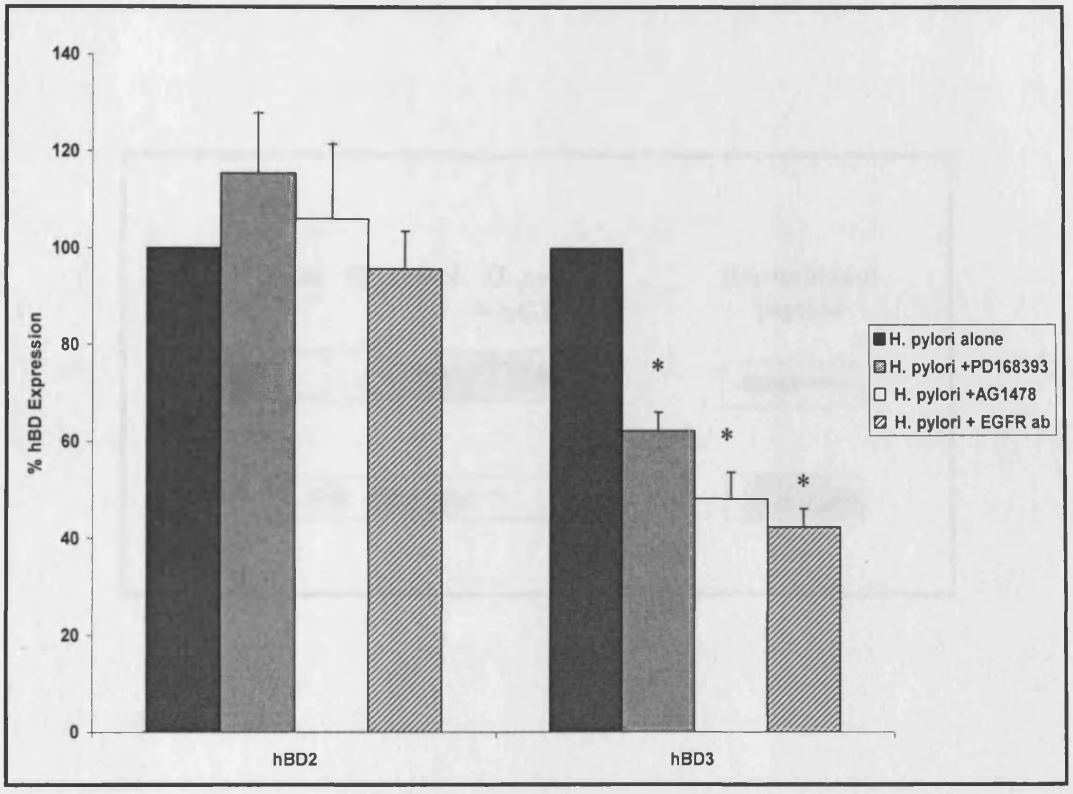

Figure 6.3: *H. pylori*-mediated hBD3 expression is dependent on EGFR signalling.

AGS cells were pre-treated with selective inhibitors for EGFR, AG1478 (3 μM), PD168393 (2 μM) or neutralising antibody (10μg/ml) for 30-60min before co-infection with *H. pylori* strain 60190. Induction of hBD2 and hBD3 mRNA was assessed 8h post-infection by RT-PCR. β-defensin levels were normalised to GAPDH and expressed as fold induction compared to uninfected control cells. Data shown were obtained from three independent experiments and results are presented as mean values ± SEM. Statistical significance was determined by t- test, * $p < 0.02$.

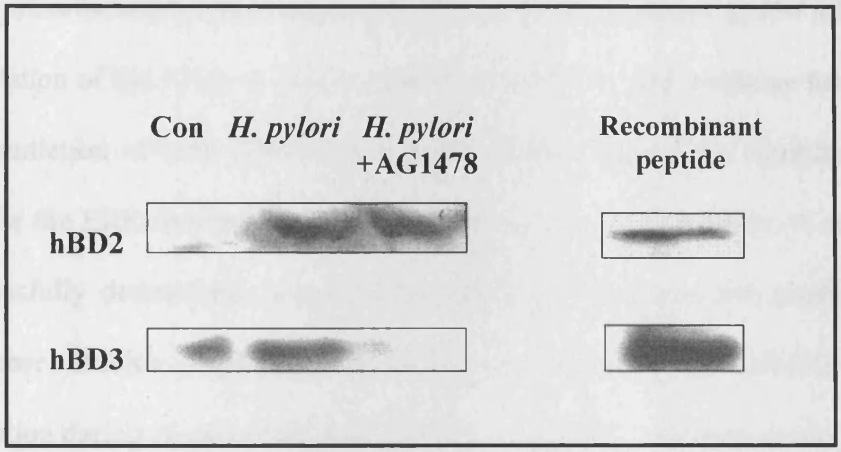

Figure 6.4: EGFR transactivation is a critical upstream signalling event in *H. pylori*-mediated hBD3 expression.

Control un-infected and 24h post-infected AGS cell lysates and supernatants were subjected to 16% Tris-Tricine SDS PAGE prior to detection of secretory hBD2 and hBD3 (cell lysate) peptides by Western Blotting. 150μg protein was loaded as determined by Bradford assay and recombinant peptides were included as positive controls. EGFR inhibitor, AG1478 (3μM) caused a significant decrease in hBD3 but not hBD2 protein levels.

6.4 Conclusions

Collectively, these experiments support the notion that transactivation of the EGFR and subsequent downstream signalling cascades may play a critical role in hBD3 gene and peptide expression with a minimal role in hBD2 regulation during *H. pylori* infection.

The elucidation of the EGF→ Ras →Raf-1→ MEK→ ERK pathway for proliferation and differentiation of cells (O'Neill and Kolch, 2004; Figure 6.2), identifies a potential pathway for the ERK-mediated hBD3 expression observed in our study. A role for EGFR was successfully determined, however, the ligand involved was not established in this study. Other studies implicate metalloprotease cleavage of HB-EGF in EGFR transactivation during *H. pylori* infection (Keates *et al.*, 2001, Wallasch *et al.*, 2002).

Keates and colleagues have demonstrated EGFR-mediated Ras activation is an upstream signalling event leading to ERK activation in *H. pylori* infected AGS cells (Keates *et al.*, 2005), which supports the established EGFR-ERK pathway engagement during *H. pylori* infection and that may also be involved in hBD3 gene regulation. Interestingly, a recent study has implicated EGFR transactivation in hBD3 gene expression in a skin model of inflammation (Sorensen *et al.*, 2005). β-defensin expression was induced in epidermal cultures which were exposed to mononuclear blood leukocytes stimulated with several PAMPs. The induction of hBD3 was inhibited by the addition of a neutralising antibody to EGFR and furthermore, the ligands responsible for the up-regulation of this inducible peptide in keratinocytes were identified as TGFα being the most potent followed by EGF, HB-EGF and amphiregulin. Unlike the study conducted by Sorensen *et al*, the present study investigated the mechanism of β-defensin induction during an infection model, which will undoubtedly involve complex molecular cross-talk and more than one signalling pathway occurring at any given time.

Some studies suggest AMPS such as LL-37 and HNPs may directly act as inducers of EGFR transactivation, possibly by mediating metalloprotienase cleavage of membrane-bound ligands (Aarbiou *et al.*, 2004, Tjabringa *et al.*, 2003). This may represent a dual role for AMPs in triggering an innate immune response in addition to being effective antimicrobials. No studies to date have suggested such a role for the β-defensin family.

More recently, not only did the transactivation of EGFR lead to increased hBD3 expression, but also induction of two other AMPs; neutrophil gelatinase-associated lipocalin (NGAL) and secretory leukocyte protease inhibitor (SLPI) was also observed in skin (Sorensen *et al.,* 2006). The EGFR-mediated up-regulation of hBD3 in this setting was activated by HB-EGF and led to an increased antibacterial activity against *Staphylococcus aureus.*

Like NF-κB-mediated inflammatory responses, I propose that transactivation of EGFR leading to production of AMPs also presents a major innate immune axis responsible for providing a sterile environment and protection against microbial invasion and colonisation during wound healing.

CHAPTER 7

Discussion

The Gram-negative bacterium *Helicobacter pylori* colonises the gastric epithelium and causes acute and chronic inflammation of the stomach (gastritis). It is strongly associated with peptic ulceration, MALT lymphoma and adenocarcinoma (Blaser, 1996). *H. pylori* possesses several virulence determinants which, together with environmental factors, are considered to affect and modulate subsequent host innate and adaptive immune responses. It is becoming increasingly evident that clinical manifestations of *H. pylori* infection are resultant from cumulative effect of multiple interactions between the bacterium and host. Antimicrobial peptides of the β-defensin family are expressed at epithelial surfaces where they play an integral role in mucosal defence (Biragyn, 2005, Ganz, 2005, Klotman and Chang, 2006, Pazgiera *et al.*, 2006, Selsted and Ouellette, 2005). Human beta defensin 1 (hBD1) is constitutively expressed through the GI tract, suggesting a surveillance-like role for this peptide, whereas hBD2 and -3 are inducible upon infection (Harder *et al.*, 2000, Harder *et al.*, 2001, O'Neil *et al.*, 1999, Wada *et al.*, 1999, Wehkamp *et al.*, 2004) and inflammation. *H. pylori* can cause modulation in the expression of all three defensins (Bajaj-Elliott *et al.*, 2002, George *et al.*, 2003). The extensive use of commercial antibiotics has led to the emergence of resistant bacterial strains that pose a major health burden. As β-defensins are endogenous antibiotics, proven to be effective in killing a range of micro-organisms, extensive research is currently being conducted in exploring the potential of β-defensins as novel therapeutic agents against infection.

Current guidelines indicate first-line therapy for treating *H. pylori* infection comprises a combination of any two antibiotics (amoxicillin, clarithromycin or metronidazole) with a proton pump inhibitor (PPI) or ranitidine bismuth citrate (RBC) (Di Mario *et al*, 2006). However, this regimen fails in 20% to 40% of patients (Vilaichone *et al*, 2006) and antibiotic resistance is one of the prominent causes for treatment failure, in addition to poor compliance, short duration of therapy, and drug-related side effects. This increase in

resistant bacterial strains has led to growing concern over effective eradication of this persistent pathogen and hence the search for novel antimicrobials.

We and others have previously highlighted the potent bactericidal activity of human β-defensin 2 and 3 against *H. pylori in vitro* (George *et al.*, 2003, Hamanaka *et al.*, 2001). To address the issue of β-defensins being bactericidal *in vivo*, experiments have shown that their antimicrobial potencies are minimally affected in the presence of acidic conditions (pH 2-4) therefore no inactivation should occur due to the low pH in the human stomach. Similarly, killing assays have been performed to determine synergistic effect not only between the β-defensins but also with other AMPs such as LL-37 and lysozyme against *H. pylori*, a potential scenario *in vivo*. This data raises the question as to "why β-defensins if so potent *in vitro*, are not able to clear the infection *in vivo*?" One may hypothesise that *H. pylori* may manipulate host anti-microbial/β-defensin levels in such a way that insufficient amount of peptides are produced that are unable to clear infection. This may be explained, in part at least, if individuals with high peptide levels show greater ability to reduce and clear infection compared to individuals who are low β-defensin producers. Recent evidence suggesting β-defensin gene copy numbers can vary between 2 and 12 (Linzmeier and Ganz, 2005) and that the copy number is the major determinant of protein produced adds credence to our suggestion. Secreted β-defensins along with other AMPs provide an antimicrobial gradient from the mucosal surface to the lumen and the amount of peptides are likely to determine the concentration of this gradient. The strength of this gradient is likely to be crucial in allowing *H. pylori* to successfully attach and attack the epithelial surface. This phenomenon provides at least one explanation for the observed variation in *H. pylori*-related diseases amongst a population of individuals with unique genotypes and therefore corresponding phenotypes.

As the host antimicrobial response is one of the initial host factors encountering pathogens at mucosal surfaces, further insight into how *H. pylori* modulates this response to its advantage was required. Therefore, the major aim of this thesis was to a) delineate the role of bacterial virulence determinants in modulating β-defensin expression and b) to identify the critical signal transduction events involved in β-defensin regulation. The latter was important as identification of major players that determine the 'robustness' of host innate antimicrobial immunity to infection may open new avenues for therapeutic intervention.

Intial studies confirmed the presence of hBD1 and -2 gene expression in gastric epithelial cells during *H. pylori* infection. Furthermore, hBD3 gene expression and both hBD2 and -3 peptide induction *via* Western Blotting in response to infection in AGS cells were demonstrated. Kinetic studies showed rapid induction of hBD2 mRNA, maximal expression was observed 6h post infection (Figure 3.2a). IL-8 also showed characteristics of an early innate immune response gene with comparable timing to hBD2, suggesting that hBD2 and IL-8 are induced rapidly to provide an acute immune response to infection. Since hBD2 is now known to also exhibit chemotactic properties for cells of the adaptive immune system, in this case, hBD2 may have a dual role *in vivo* by recruiting other immune cells in addition to being an antimicrobial peptide.

In order to address the first main hypothesis of this investigation, isogenic mutants of *H. pylori* strain 60190 were utilised to determine the potential role of bacterial virulence factors in modulating epithelial β-defensin expression. Induction of hBD2 was found to be dependent upon an intact bacterial secretion system (Figure 3.4). The use of various bacterial strains including *Cag*PAI-ve clinical strain, J150, verified this result (Figure 3.3). Published work with isogenic mutants (including *CagE)* have implicated a crucial role for an intact secretion system, but not the translocation of bacterial CagA, in eliciting activation of the immediate early response transcription factor, NF-κB and subsequent gene

expression of various proinflammatory molecules such as IL-8, IL-1β and TNF-α (Maeda *et al*, 2001, Crabtree *et al.*, 1999, Fischer *et al.*, 2001, Munzenmaier *et al.*, 1997, Naumann, 2000, Selbach *et al.*, 2002, Viala *et al.*, 2004). One study to date has documented a role for *H. pylori Cag*PAI in the induction of hBD2 mRNA (Wada *et al.*, 1999). In the present study, a minimal role for both CagA and VacA virulence factors was found in modulating epithelial antimicrobial responses despite the dramatic phenotype changes caused by both toxins. This suggests some divergence in epithelial signalling pathways that dictate innate immune responses *versus* cytoskeletal changes caused by CagA and VacA (humming bird phenotype and vacuolation respectively). Exclusion of the injected CagA protein as a bacterial factor responsible for β-defensin gene expression led us to speculate the nature of the bacterial constituent responsible for instigating epithelial innate immune responses. Further delineation of these events is likely to be beneficial for successful future drug design.

Prior to investigating mechanism(s) regulating epithelial β-defensin expression, it was important to establish hBD2 induction in our cell system was directly due to the bacterium and not a secondary effect to production of IL-1β, a well-known agonist for hBD2. Minimal effect of IL-1Ra on bacterial-mediated defensin expression was observed (Figure 4.8). This finding suggests the bacteria are directly exploiting host innate signalling pathways to modulate epithelial defensin expression. In spite of this, it is likely that IL-1β paracrine effects following release from inflammatory cells would be an additional stimulus to epithelial cell hBD2 expression *in vivo* in the gastric mucosa.

Experiments investigating expression and regulation of hBD2 and -3 suggested different kinetics of expression for the two antimicrobials. Compared to the rapid (6h) expression of hBD2, hBD3 mRNA up-regulation was slower with levels increasing between 12-24h post-infection. This finding provided the first indication of differential regulatory mechanism(s)

for hBD2 and hBD3 gene expression. The differing kinetics suggests provision of a more global host antimicrobial blanket rather than a potent short burst may be more beneficial to the host in protecting itself against an invading microbe. Physiologically, overlapping antibacterial functions of hBD2 and -3 is likely to provide more sustained and effective antimicrobial barrier and it would be interesting to study this further in the context of how lysozyme and LL-37 impact and modify the strength of this protective shield.

Several binding sites for NF-κB and AP-1 in hBD2 promoter suggest multiple signalling pathways can impact on its gene regulation and their involvement is likely to differ depending upon the initiating stimulus (Krisanaprakornkit *et al.*, 2000, Krisanaprakornkit *et al.*, 2002). As eukaryotic cells commonly respond to extracellular signals via the activation of the MAP Kinase pathways and NF-κB, it was pertinent to study these signalling pathways and in particular, a specific aim of this study was to delineate the signal transduction events involved in the regulation of *H. pylori*-epithelial defensin cross-talk. I hoped that this study may provide further insight into signalling events that may be considered as future therapeutic targets for modulating host immunity to infection.

Although the transcription factor NF-κB has been implicated in both IL-1β and *H. pylori*-mediated hBD2 gene expression, very little is known about the regulation of hBD3 and therefore it was sought to identify the regulatory mechanisms involved in hBD3 expression during *H. pylori* infection. A minimal role for NF-κB in *H. pylori*-mediated hBD3 expression was found (Figure 4.7), which was in agreement with promoter analyses revealing no NF-κB binding site in the promoter region. The lack of involvement of NF-κB and thus lack of involvement of NOD1 in regulating hBD3 may help some way to explain the varying kinetics observed for NF-κB-dependent hBD2 expression compared to hBD3 and is clearly a parameter defining the two subsets of innate immune genes.

Owing to *H. pylori* being an extracellular pathogen, the trans-membrane TLRs seem to be ideal candidate host receptors to be involved in epithelial bacterial sensing. Surprisingly, this family in general is not being exploited by *H. pylori* at the epithelial surface. Not only gastric epithelium but intestinal epithelium is also hypo-responsive to Gram-negative LPS (Eckmann, 2006). As the gastrointestinal tract is in continuous contact with microbes (particularly the colon), one may speculate that hyporesponsivenss to LPS at the mucosal surface is a strategy employed by the host ensuring tissue homeostasis as otherwise, continuous detection of LPS from resident microflora would result in constant inflammatory activation, a scenario surely detrimental to health. At the same time, low biological activity of *H. pylori* LPS clearly suggests that the bacteria itself has evolved to minimise its detection by the host, a modification that must aid *H. pylori* in being persistent in asymptomatic individuals. Similarly, the bacterial flagellin protein has evolved with sequence changes that allow it to escape detection by host epithelial TLR5 and yet maintain motility (Andersen-Nissen *et al.*, 2005), a crucial process that the bacteria must undergo to successfully cross the protective mucus lining prior to epithelial adherence. Further studies investigating bacterial modifications that allow enteropathogens to successfully colonisation the GI tract are eagerly awaited.

In order to delineate the signal transduction pathways mediating hBD expression, it was imperative to clarify a role if any, for TLRs in mediating NF-κB activation leading to hBD2 gene expression during *H. pylori* infection. By disruption of host cell signalling complexes integral to TLR and IL-1β transduction and subsequent inhibition of downstream signalling, no effect of these pathways on bacterial-mediated hBD2 expression was observed (Figure 4.9). This clearly demonstrated that *H. pylori* has evolved mechanism(s) that allow it to circumvent any recognition and activation by epithelial TLRs.

A second family of PRRs has been described to play crucial role in epithelial host innate immune responses to infection, the cytoplasmic NOD proteins (Inohara *et al.*, 2002, Philpott and Girardin, 2004, Strober *et al.*, 2006). Although NOD1 but not NOD2 mRNA was detected in AGS gastric epithelial cells (Figure 4.10), expression of both NOD1 and NOD2 receptors is present in the gastric epithelium of *H. pylori*-positive individuals (Rosenstiel *et al.*, 2006) and importantly, a significant role for NOD1 in the detection of *H. pylori* by epithelial cells has been reported (Viala *et al.*, 2004). NF-κB activation was dependent upon the presence of an intact *cag*PAI, indicating the type IV secretion system is required for the intracellular delivery of bacterial iE-DAP. As the TLRs were ruled out by preceding experiments, it was hypothesised that NOD1 may play a role in *H. pylori*-mediated β-defensin gene regulation. Experiments utilising siRNA for NOD1 implicated NOD1 engagement in *H. pylori*-mediated hBD2 expression (Figure 4.12 and 4.13). Since hBD2 expression was found to be exclusively dependent upon an intact secretion system, this fits in with NOD1 receptor being responsible for its induction as *H. pylori* rarely invades cells and therefore the type IV secretion apparatus would be required for intracellular translocation of NOD1 ligand, iE-DAP. Any bacteria freely residing in the gastric mucus layer would remain in their niche undisturbed without triggering host innate immunity allowing persistent colonisation. However, if adherent *Cag*PAI+ve bacteria became intimate with epithelial cells, it is possible that peptidoglycan from cell wall may 'leak' through to host cells where the tri-peptide motif may bind NOD1 and initiate signals to activate the host innate immune response. Ultimately, the production of specific antibacterial agents such as β-defensins would be detrimental to the pathogenic micro-organism but even without direct invasion of epithelial cells, the bacteria are capable of inducing β-defensin expression. One may also speculate that that the induction of AMPs could be beneficial to persistent pathogens, as any transient infections caused by other

bacterial species would be effectively killed by AMPs as they may not exhibit sophisticated mechanisms to subvert innate immunity.

Furthermore, murine experiments suggest a role for NOD1 in mediating mBD4 (an orthologue of hBD2) expression *in vivo* (Figure 4.14). It is a crucial step in the development of β-defensins as potential therapeutics to ascertain their *in vivo* functional relevance by complementing *in vitro* investigations. It is a fundamental finding to establish the PRR mediating production of theses AMPs *in vivo*, so that future efforts may be targeted accordingly. These findings provide evidence for the primary hypothesis in this study by linking impaired β-defensin expression to increased colonisation observed in the NOD1 KO mice (Viala *et al.*, 2004). It is entirely possible that low mBD4 expression levels contribute to reduction in antimicrobial function, leading to the observed increase in colonisation in the murine stomach. In conclusion, these data highlight a key role for NOD1 in modulating host anti-microbial function *in vivo*.

As differential kinetics and contribution of NF-κB for hBD2 compared to hBD3 were observed, it was not expected that both peptides are regulated by the same mechanism. So, to delineate signal transduction events mediating hBD3 expression during *H. pylori* infection another set of signalling pathways which play a vital role in infection and inflammation, the MAPKs were investigated. The activation of MAPK pathways during *H. pylori* infection has been well studied (Keates *et al.*, 1999, Meyer-ter-Vehn *et al.*, 2000, Mitsuno *et al.*, 2001, Naumann *et al.*, 1999, Naumann and Crabtree, 2004, Torok *et al.*, 2005). Prior to establishing a role for the MAPK in β-defensin gene regulation, it was pertinent to confirm the activation of MAP kinase pathways in our co-culture system (Figure 5.1 and 5.2). In agreement with previous findings, activation of all three MAPK pathways was observed and the use of pharmacological inhibitors implicated all three pathways to varying degrees in both hBD2 and hBD3 gene regulation. RT-PCR analyses

for hBD2 and IL-8 indicated discrete signalling mechanisms which were stimuli-specific are involved in the regulation of this subset of innate immune genes. Luciferase promoter constructs for the β- defensins indicated that ERK pathway seemed to play a role in both cytokine and bacterial-mediated hBD2 and -3 expression, whereas p38 pathway was not implicated in cytokine-mediated hBD3 expression (Figure 5.4 and 5.5). Any firm conclusions by singling out any particular pathway in β-defensin expression were not able to be established.

By selectively activating individual MAPK pathways (utilising conditional kinase cell-lines) in the absence of any other signalling event ERK activation alone induced hBD3 but not hBD2 expression. This important finding suggests that ERK pathway must synergise or cross-talk with other pathways which are likely to be activated during infection to modulate hBD2 expression. These studies so far implicated NOD1-dependent NF-κB activation and the JNK pathway in hBD2 gene regulation. In contrast to hBD2, hBD3 expression was NOD1 independent but ERK- and JNK pathway-dependent. Like preceding findings in this study the involvement of ERK yielded another difference in the regulation of hBD2 compared to hBD3, which provided a clue as to what upstream effectors may be regulating hBD3. As the ERK pathway can be activated by *cag*PAI-ve strains (Naumann and Crabtree, 2004), it was hypothesised that the EGF receptor is the most likely candidate upstream of the ERK pathway (shown by Keates *et al.,* 2005) and hBD3 expression. Indeed, a role for EGFR transactivation in hBD3 mRNA and peptide expression was identified (Figures 6.3 and 6.4), in this model of infection. I propose EGFR may also function as a critical regulator of a subset of innate response genes with hBD3 a prominent member.

The molecular events leading to EGFR transactivation during infection were not investigated in the present study although metalloprotease cleavage of HB-EGF may play a

role (Wallasch *et al.*, 2002). Importantly, recent advances in therapy for cancer have targeted EGFR signalling and in particular utilised pharmacological inhibitors and monoclonal antibodies at the clinical level (Mendelsohn, 2002). This would be a significant treatment in the subset of *H. pylori*-infected individuals which are suffering form gastric cancer. However, the targeting of the EGFR pathway would prevent production of antimicrobial hBD3, which may provide critical protection in wound healing phase of infection by killing any opportunistic pathogenic micro-organisms whilst the epithelial barrier is most vulnerable. Recent advances are being made into investigating the killing mechanisms of AMPs to use them as anti-cancer therapy (Papo and Shai, 2005), as damaging the cell membrane would be an effective way of eliminating cancerous cells. However, further studies into these potential applications are required to determine the molecular mechanisms underlying anti-cancer activity.

Despite the effectiveness of β-defensins in killing *H. pylori,* the question remains how this gastric pathogen is capable of persisting in the presence of host immune responses. In addition to the virulence factors and host genetic factors discussed earlier, there are several other determinants contributing to the success of *H. pylori* as a persistent coloniser of the human stomach. An essential colonisation factor is the acquisition of nutrients, namely iron, as mutants lacking in the expression of ferritin are unable to colonise (Waidner *et al.,* 2002). This allows the bacterium to survive beyond the initial colonisation period where it utilises urease to alkalinise the acidic microenvironment of the stomach and establish its niche. Also, the production of its own antimicrobial agent highlights this bacterium's sophistication as this ability would enable the clearance of other potential competitive pathogens (Fu *et al.*, 2004).

Importantly, previous work defining a role for the murine BD1 utilising deficient mice has shown increased susceptibility to certain pathogens (Morrison *et al.*, 2002, Moser *et al.*,

2002) indicating these peptides are not necessarily redundant in their nature and are critical contributors of host defence against infection *in vivo*. Another murine study investigating β-defensin expression after vaccination with a construct encoding *H. pylori* urease enzyme observed an increase in mBD1 in the stomach of immunised mice (Hatzifoti *et al.*, 2006). In addition to the adaptive immune system being protective by production of *H. pylori*-specific antibody and T-cell proliferation, it seems that the induction of mBD1 may aid in limiting colonisation at the mucosal surface thus conferring protection through host innate immune responses. In this case, not only is defensin production a first line defence, it appears to be involved in a more complex global immune response intertwining innate and adaptive immunity. These studies collectively draw attention to the importance of β-defensins in host defence *in vivo* and complement *in vitro* work implicating AMPs as significant components of innate immunity.

In conclusion, this study has shown that dynamic modulation of host epithelial anti-microbial immunity occurs in response to *H. pylori.* The bacterium indulges in direct epithelial cross-talk exploiting host responses in order to persistently colonise the gastric epithelium. The distinct regulatory mechanisms and overlapping functions of hBD2 and -3 try to provide an antimicrobial shield through out the inflammatory and wound healing phase of infection, in individuals infected with more virulent strains and low defensin expression the antimicrobial shield may prove to be insufficient leading to greater persistent colonisation culminating in greater clinical manifestations and complications.

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Host anti-microbial response to *Helicobacter pylori* infection

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Intestinal Innate Immunity to *Campylobacter jejuni* Results in Induction of Bactericidal Human Beta-Defensins 2 and 3

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*CRITICAL REGULATORS OF β-DEFENSINS DURING HELICOBACTER PYLORI INFECTION**

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